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Structure-guided insights into the functions of CHIKV nsP2

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Structure-guided insights into the functions of CHIKV nsP2

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TABLE OF CONTENTS

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The thesis is based on the three original papers, listed below:

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II. Law, Y.-S., **Wang, S**., Tan, Y. B., Shih, O., Utt, A., Goh, W. Y., Lian, B.-J., Chen, M. W., Jeng, U.-S., Merits, A., & Luo, D. (2021). Interdomain Flexibility of Chikungunya Virus nsP2 Helicase-Protease Differentially Influences Viral RNA Replication and Infectivity. Journal of Virology, 95(6), e01470–20. https://doi.org/10.1128/JVI.01470-20.

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Author contributions:

I. I was involved in the experimental design and the construction of a set of *trans*-replicase plasmids containing identified adaptive mutations and pseudoreversion.

II. I was responsible for the experimental design and the construction of CHIKV *trans*-replicases with deletions and insertions in the linker region of nsP2, and I performed all related experiments.

III. I designed and constructed the majority of the plasmids, performed all the experiments described in the publication, analyzed the results of these experiments, and wrote the manuscript.

LIST OF ABBREVIATIONS

AUD	alphavirus unique domain
ATP	adenosine triphosphate
BHK	baby hamster kidney
CD2APCD2	Associated Protein
CHIKV	chikungunya virus
CHIKF	chikungunya fever
CMV	human cytomegalovirus
CP	capsid protein
CPE	cytopathic effect
CPV	cytopathic vacuole
CSE(s)	conserved sequence element(s)
DI	defective interfering
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
dsRNA	double-stranded RNA
EEEV	Eastern equine encephalitis virus
EGFP	enhanced green fluorescent protein
EIF2	eukaryotic translation initiation factor
EILV	Eilat virus
ER	endoplasmic reticulum
FDA	Food and Drug Administration (USA)
FHL1	four and a half LIM domains 1
FXR	protein from fragile X syndrome family
G	genomic (RNA or promoter)
G3BP(s/1/2)	Ras-GTPase-activating protein (GAP)-binding protein(s/1/2)
GAGs	glycosaminoglycans
GTP	guanosine-5'-triphosphate
GTase	guanylyltransferease
HSC70	heat-shock protein 70
HS	heparan sulphate
HVD	hyper variable domain
ICA	infectious centre assay
IFN	interferon
IFIT1	interferon-induced protein with tetratricopeptide repeats 1
IRES	internal ribosome entry site
JAK-STAT	Janus kinase-signal transducer and activator of transcription
LDLRAD3	low density lipoprotein receptor class A domain containing 3
L-SIGN	liver-specific SING
MAYV	Mayaro virus
MTase	N7-guanine-methyltransferase
NAP1L1	nucleosome assembly protein 1-like 1
NC	nucleocapsid

NLS	nuclear localization signals
ns	nonstructural
nsP(s)	nonstructural protein(s)
nsP2h	nsP2 helicase region
nsP2p	nsP2 protease region
NTP	nucleoside triphosphate
NTPase	nucleotide triphosphatase
NTD	N-terminal domain
NRAMP	natural resistance-associated macrophage protein
ONNV	o'nyong-nyong virus
ORF(s)	open reading frame(s)
PEKR	PKR-like ER resident kinase
PKR	protein kinase R
PS	packaging signal
RC	replication complex
Rin	Rasputin (G3BP ortholog in mosquitoes)
RRM	RNA recognition motif
RRV	Ross River virus
RSE	repeated sequence element
RTPase	RNA triphosphatase
SG	subgenomic (RNA or promoter)
SH3KBP1	SH3 domain-containing kinase-binding protein 1
SINV	Sindbis virus
SFV	Semliki Forest virus
TF	trans frame protein
ts	temperature sensitive
U2OS	human osteosarcoma cell line
U2OS ΔΔ	U2OS-derived double-null ΔΔ G3BP1/2-knockout cells
Ubi	polyubiquitin promoter of *Aedes aegypti* mosquito
UPR	unfolded protein response
UTR	untranslated region
VLDLR	very low-density lipoprotein receptor
VEEV	Venezuelan equine encephalitis virus
YFV	yellow fever virus
wt	wild-type

INTRODUCTION

Alphaviruses are small, spherical, enveloped viruses with positive-strand RNA genomes. These viruses are responsible for many human and animal diseases, and large outbreaks and epidemics have occurred in recent decades. Alphaviruses are widespread throughout the world and are found on all continents except Antarctica. Most known alphaviruses are arboviruses that infect vertebrate hosts via blood-sucking arthropod vectors, mainly mosquitoes (most commonly *Aedes* and *Culex* mosquitoes). Alphaviruses include important human pathogens such as chikungunya virus (CHIKV), o'nyong-nyong virus (ONNV), Sindbis virus (SINV), Ross River virus (RRV) and Eastern equine encephalitis virus (EEEV), and new pathogens such as Mayaro virus (MAYV) are emerging. These viruses can cause arthritic diseases or encephalitis in humans and animals, and they remain a worldwide threat due to urbanization, the spread of vector-competent mosquito species, and adaptations to new arthropod vectors.

One of the most medically important alphaviruses is CHIKV, which can cause chikungunya fever (CHIKF), an acute febrile disease typically accompanied by a rash and by debilitating and chronic arthralgia that can persist for months or even years. Since its first documented outbreak in 1952 in Tanzania, CHIKV has caused millions of cases in more than 50 countries. CHIKV is considered a re-emerging pathogen of global importance, but there are no licensed drugs available for treating CHIKV infections. The high pathogenicity of CHIKV and its ability to cause chronic disease are likely due to specific characteristics such as its replication and/or interactions with hosts.

The genomic RNA replication of alphaviruses depends on RNA synthesis and modification carried out by nonstructural proteins (nsPs) encoded by viral RNA. Four nsPs are encoded by the alphavirus genome, and each has multiple unique functions that are necessary for alphavirus RNA replication or other crucial processes in the infection cycle. Among the nsPs, nsP2 is considered the main driving force and master regulator of RNA replication, with four known enzymatic activities involved in the replication process. The N-terminal region of nsP2 (nsP2h) acts as an RNA helicase, nucleoside triphosphatase (NTPase), and RNA 5′ γ-phosphatase (RTPase). However, its RNA helicase activity is displayed only in full-length nsP2, i.e., the C-terminal region of nsP2 is also necessary. On its own, the C-terminal region of nsP2 (nsP2p) exhibits protease activity; it is a cysteine protease responsible for sequentially cleaving ns-polyproteins (the precursors of nsPs) to form functional replication complexes. Interestingly, some of these cleavages can be performed by nsP2p, while one of them requires full-length nsP2, i.e., the N-terminal region is also necessary. Thus, these two seemingly different regions of nsP2 are functionally coupled and affect each other's activities. Moreover, nsP2 has been shown to be a determinant for the binding of viral RNA to RNA replication complexes and for its packaging into virions. nsP2 also modifies the host cellular environment both by activating antiviral responses and by counteracting them through multiple mechanisms, for example, by shutting off host

transcription. The multifunctional nature of nsP2 and its central role in alphavirus infection have made nsP2 an object of multiple studies by many research teams. However, as the protein is complex and has a large number of different yet linked functions, additional studies are needed to further elucidate its unknown facets and how known and novel functions are coordinated.

The aim of this study was to enhance the understanding of the function of CHIKV nsP2 in viral RNA replication, with a specific focus on its N-terminal domain and the flexible linker between the N- and C-terminal domains. We identified the crucial residues that form stacking interactions between nsP2h and the conserved 3' end 14 nucleotides of the CHIKV genome. Disruption of these interactions was found to have detrimental effects on viral RNA replication. Similarly, the NTP binding site was crucial for CHIKV replication. The flexible interdomain linker that connects the N-terminal helicase and C-terminal protease regions, was also found to be essential for CHIKV replication, but small changes, such as the deletion of one amino acid residue and the insertion of up to 10 amino acid residues, were tolerated without significantly impacting viral replication. However, the deletion of three or five amino acid residues was lethal to CHIKV. Furthermore, the insertion of G3BP/Rin binding motifs into flexible regions of nsP2 (in the linker region or between different domains of nsP2p) was not only tolerated but able to support the replication of CHIKV mutants in both mammalian and insect cells. The same insertion also restored the ability of viruses lacking G3BP binding motifs in nsP3 to replicate in mammalian (BHK21) cells, whereas alone, the lacking of the G3BP binding motifs in nsP3 is lethal for CHIKV. These findings demonstrated that the CHIKV G3BP binding motifs can be transferred from its natural position in the C-terminal region of nsP3 to nsP2 and remain functional.

Overall, these findings enhanced our understanding of the role of nsP2 in the alphavirus replication process and allowed us to further investigate the coordination of the multiple enzymatic and nonenzymatic activities of nsP2 from both structural and functional perspectives. Importantly, the amino acids that were identified as crucial in nsP2 could be used as potential targets for antiviral compounds, while mutations of these residues could be exploited to generate attenuated vaccine candidates. In addition to the globular domains, the flexible regions of nsP2 were also found to be highly important, and their roles in processes involving nsP2 deserve specific study.

1. LITERATURE OVERVIEW

1.1. Alphaviruses

Alphaviruses are enveloped, positive-sense RNA viruses that belong to the *Alphavirus* genus in the *Togaviridae* family (https://ictv.global/taxonomy). To date, 32 species of alphaviruses have been officially recognized. The alphaviruses that infect warm-blooded animals can be divided into two groups, Old World and New World alphaviruses, based on their geographical distribution and the diseases they cause. Chikungunya virus (CHIKV), o'nyong-nyong virus (ONNV), Mayaro virus (MAYV), Ross River virus (RRV), Sindbis virus (SINV), and Semliki Forest virus (SFV) are Old World alphaviruses. In their vertebrate hosts, they mainly cause fever, rash, and polyarthralgia. However, it is worth noting that in mouse models, several Old World alphaviruses (such as certain strains of SINV and SFV) also result in encephalitic syndromes, which are more typical of New World alphaviruses. This property is reflected in the names of these viruses, such as Eastern equine encephalitis virus (EEEV) and Venezuelan equine encephalitis virus (VEEV).

The majority of known alphaviruses are arboviruses, which means that they are transmitted among vertebrate hosts by hematophagous arthropod vectors, such as mosquitoes, ticks, and biting flies. Although some alphaviruses can be transmitted by other means (for example, in the form of infectious aerosols), horizontal transmission via mosquito vectors is by far the most common route for these viruses. In the transmission cycle, the vector becomes infected by feeding on a viremic blood meal, after which the virus replicates in the mosquito body and disseminates to the salivary glands. Ultimately, viruses are injected into the vertebrate host via the saliva of the vector during blood feeding (1). The vertebrate hosts of alphaviruses include mammals, birds, reptiles, and fishes (2). Table 1 lists some vectors and hosts of medically relevant alphaviruses.

Table 1. Vectors, hosts, and symptoms of pathogenic alphaviruses

Alpha-virus	Confirmed or putative mosquito vectors	Confirmed or putative reservoir hosts	Symptoms in humans
CHIKV	*Aedes aegypti; Aedes albopictus* (3),	Nonhuman primates (4)	Fever, polyarthralgia, rash, headache Chronic symptoms in up to 80% of patients
ONNV	*Anopheles funestus, Anopheles gambiae* (5)	Unknown	Similar to CHIKV (usually milder) with addition of cervical lymphadenitis
MAYV	*Haemagogus janthinomys* (6) *Aedes. Aegypti* (7)	Nonhuman primates (8)	Similar to CHIKV, mostly milder
RRV	*Culex annulirostris, Aedes vigilax* (9)	Marsupials (10)	Fever, rash, headache

Table 1. Continuation

Alpha-virus	Confirmed or putative mosquito vectors	Confirmed or putative reservoir hosts	Symptoms in humans
SFV	*Aedes* subspecies (11).	Small mammals, birds, nonhuman primates	Generally considered to be nonpathogenic in humans, occasionally causing myalgia and polyarthralgia; encephalitis can be induced in mice (12)
EEEV	*Culiseta melanura* (13) *Culex erraticus* (14)	Passeriformes birds (13)	Febrile or neurological disease, including meningitis and encephalitis.

As in the case of other arboviruses, three commonly known circulation cycles have been described for alphaviruses: the enzootic cycle, the rural epizootic cycle, and the urban epidemic cycle (Figure 1). For instance, in nature, CHIKV circulates between nonhuman primates and arboreal, canopy-dwelling *Aedes* species mosquitoes in the enzootic cycle. However, in the urban cycle, CHIKV circulates between anthropophilic mosquito vectors (mainly *Aedes aegypti* and *Aedes albopictus*) and human hosts (15).

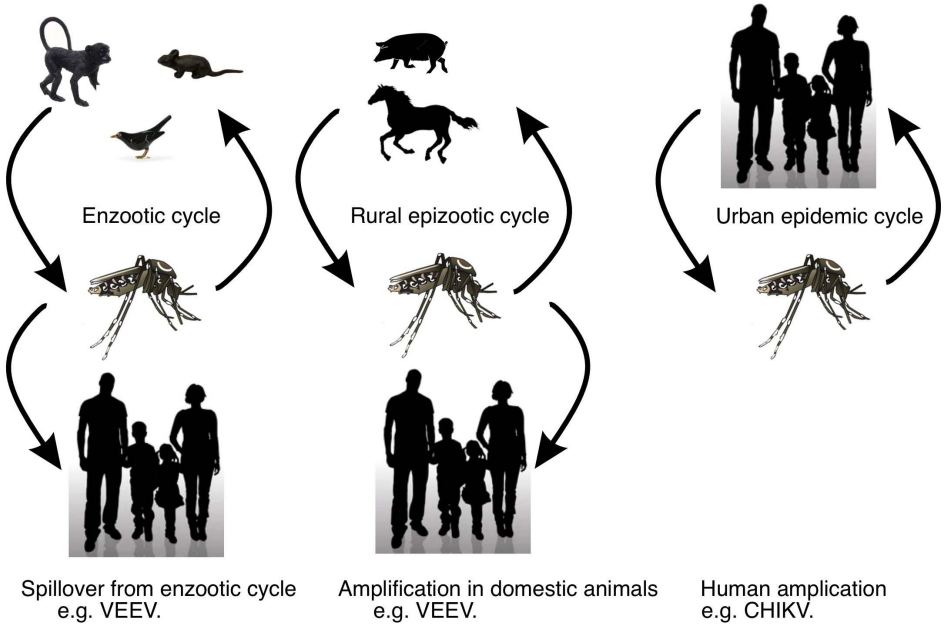

Figure 1. The circulation cycles of alphaviruses. Humans can be infected with CHIKV by spillover when entering enzootic habitats (enzootic cycle) or when contacting domestic animals (rural epizootic cycle); in these situations, the number of human cases is usually low. However, CHIKV can also be amplified in humans during the urban epidemic cycle, leading to massive outbreaks.

In terms of morbidity, one of the most pathogenic alphaviruses is CHIKV. There have been massive outbreaks of CHIKV since it was first isolated in 1953 in Tanzania (16); however, it is highly likely that CHIKV outbreaks also occurred earlier but were mistakenly attributed to dengue virus (a flavivirus causing relatively similar symptoms). Since its isolation, CHIKV outbreaks have been repeatedly reported in Africa and Asia (17). In 2004, CHIKV caused approximately 5000 reported cases in Kenya and Comoros. The outbreak spread to La Réunion and the other islands in the Indian Ocean (18). Later, CHIKV was exported to Europe, the Americas and Asia via infected travelers, leading to outbreaks in Italy, France and Asia (19–21). Massive outbreaks occurred in Central and South America in 2013–2014 (22), and CHIKV became endemic in these areas. By April 2023, CHIKV had caused millions of documented cases in more than 50 countries (Figure 2), was recognized as an important re-emerging pathogen and was listed as a causative agent of major neglected tropical diseases by the WHO (https://www.who.int/health-topics/neglected-tropical-diseases#tab=tab_1). Additionally, CHIKV infections are typically underreported for various reasons, including poor availability of medical services in the regions where CHIKV is endemic and the similarity of its symptoms to those of dengue, malaria, and many other tropical diseases. Furthermore, CHIKV shares more than 80% sequence identity with its closest relative, ONNV. ONNV is widespread in Africa and causes symptoms similar to those of CHIKV infection. Nevertheless, it is a different virus and is transmitted by *Anopheles* and not *Aedes* mosquitoes (23, 24).

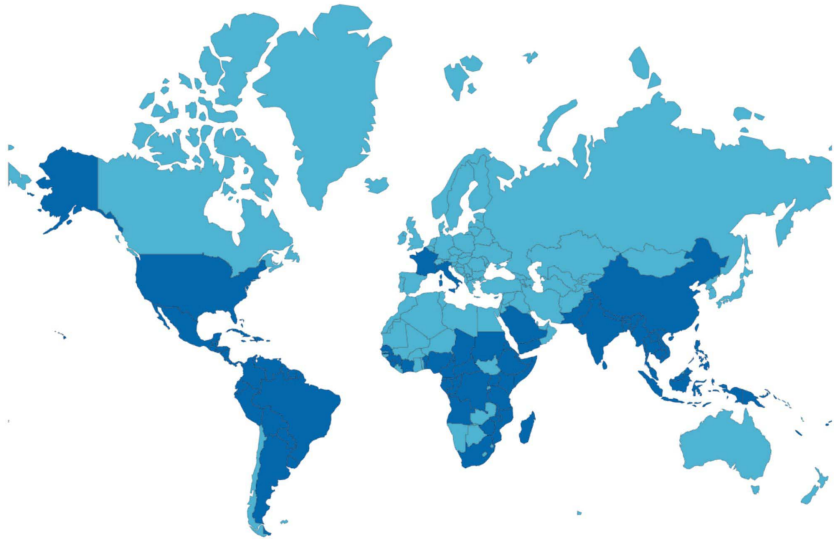

Figure 2. Worldwide spread of CHIKV (as of April 30, 2023). Countries and territories where locally transmitted CHIKV cases have been reported are shown in dark blue (https://www.cdc.gov/chikungunya/geo/index.html).

The incubation time of CHIKV infection in humans is estimated to be 2–10 days. The symptoms of CHIKV infection include fever, rigor, headache, rash, and severe joint pain. Originally, CHIKV infection was believed to be rarely fatal, and the main problem was considered to be chronic arthralgia, which can persist for months to years (25). However, recent data from South America suggest that the fatality of CHIKV infection has been underestimated, and as many as 0.13% of CHIKV infections result in death (26). There are no approved drugs for treating CHIKV infections, but the VLA1553-CHIKV live-attenuated vaccine was recently approved by the FDA in November 2023 for the prevention of disease caused by CHIKV (27).

To date, alphaviruses are among the most concerning emerging mosquito-borne viruses worldwide because of the wide distribution of mosquito vectors capable of transmitting alphaviruses and their ability to adapt to new vectors. For instance, studies have shown that MAYV, RRV and, to a degree, even ONNV can use *Aedes aegypti* and/or *Aedes albopictus* as competent vectors, even though these mosquitoes are not natural vectors of those viruses. Studies have also revealed that alphaviruses can adapt to new vectors in experimental settings (28–32). Unfortunately, the same occurs in nature. The best known example is, again, CHIKV, which has adapted to *Aedes albopictus* by acquiring the mutation A226V in the E1 glycoprotein, leading to massive outbreaks in La Réunion (3). In addition, coinfection with arboviruses increases the risk of disease. For example, coinfections of CHIKV, Zika, and dengue viruses have been reported during the Zika virus epidemic in Colombia (33). Consequently, understanding the transmission mechanisms of alphaviruses and developing diagnostic tools to facilitate distinguishing among different viruses (i.e., avoiding cross-reactivity) are important directions for research and diagnostic development.

1.1.1. Alphavirus virion and genome organization

The virions of alphaviruses are spherical and approximately 70 nm in diameter (Figure 3) (34). The outermost protein shell of the virion consists of 80 spikes formed by trimers of the heterodimeric glycoproteins E1 and E2 (35,36). The ectodomains of E1 and E2 extend into the membrane envelope, which is derived from the host cell membrane (37). The C-terminal cytoplasmic regions of E1 and E2 interact with the capsid protein (CP) (38), enclosing the genomic (G) RNA.

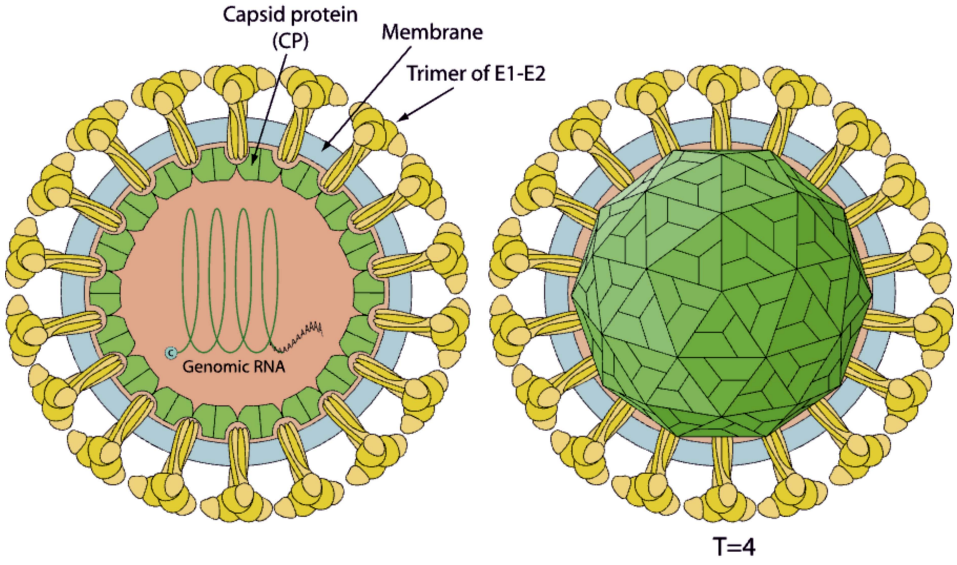

Figure 3. The structure of the alphavirus virion. The enveloped virion is 65–70 nm in diameter. The genomic RNA is enclosed in a capsid formed by 240 copies of the capsid protein. The capsid is surrounded by a lipid bilayer with embedded glycoprotein spikes. (https://viralzone.expasy.org/625)

The genome of alphaviruses is a positive-strand RNA approximately 12 kb in length (39). The genomic (G) RNA has a 5' cap 0 structure (7-methyl-GpppA) and a 3' poly(A) tail. There are two open reading frames (ORFs) in G RNA: the first ORF is translated from G RNA and encodes a nonstructural polyprotein (P1234) or two nonstructural polyproteins (P123 and P1234); the latter occurs due to the opal stop codon at the end of the region encoding nsP3. The second ORF is translated from subgenomic (SG) RNA made in infected cells and encodes precursors of viral structural proteins, namely, CP, glycoproteins (E1, E2, and E3), and 6K or the transframe (TF) protein synthesized using a ribosomal frameshift (2,40) (Figure 4). In addition to the ORFs, there are three untranslated regions (UTRs) in the alphavirus genome: the 5' UTR and the 3' UTR, which are located at the ends of the genome, and the intergenic region, which is located between the ORFs. The UTRs of alphaviruses contain conserved *cis*-acting sequence elements (CSEs) that are important for viral synthesis (41,42). For instance, the first 44 nt of the 5' UTR represent CSE1, a multifunctional element with a crucial role in viral RNA replication and the ability to interfere with the activities of IFIT1, an interferon-stimulated protein; to prevent the IFIT1-mediated blockade of alphavirus genome translation (43). In addition to CSEs, the genomes of alphaviruses contain other RNA elements that mediate viral replication and modulate the stability of viral RNAs. For example, repeated sequence elements (RSEs) are approximately 40–60 nt in length and are located in the 3' UTR. In CHIKV, the presence of RSEs has positive effects on viral fitness

in mosquitos but is negatively correlated with fitness during infection in mammalian models (44). This contradictory role is characteristic of many genetic elements and proteins of alphaviruses and reflects the need to infect different organisms and the different goals of viruses in their vertebrate hosts (high-titer viremia is needed to infect mosquito vectors) and in vectors (dissemination without significant damage is needed for transmission to vertebrate hosts).

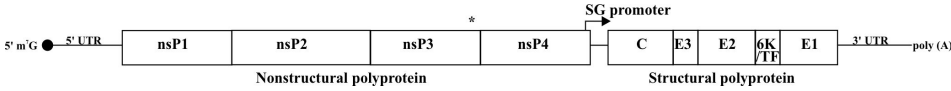

Figure 4. The organization of the genome of alphaviruses. The alphavirus genome contains two ORFs, one that encodes nonstructural polyproteins and one that encodes structural polyproteins. There is a cap 0 structure (7-methyl-GpppA) and a poly (A) tail at the 5' UTR and the 3' UTR, respectively. * designates an opal stop codon that is present in the first ORF of the majority of alphaviruses.

1.2. Alphavirus infection cycle

1.2.1. Binding, entry, and replication

For alphaviruses to initiate infection, they must bind to and enter permissive cells, which allow their replication. The process of binding and entry is not fully understood, and the literature concerning this process is rather contradictory. In older literature, the identities of alphavirus receptors and their roles were either unknown or very vague; in contrast, in the past five years, many molecules that are involved in alphavirus entry have been identified, and structural information on how viruses interact with these molecules has become available. However, obtaining a complete picture of entry is still challenging, partly because of differences among alphaviruses and partly because of their ability to infect both vertebrate and mosquito cells.

Alphaviruses use attachment factors, which allow the virus to make direct contact with the target cell; this interaction facilitates the subsequent receptor binding and internalization of viruses (45). Heparan sulfate (HS) is a sulfated polysaccharide glycosaminoglycan (GAG) expressed on cell surfaces as a component of extracellular matrices and is utilized by some alphaviruses as an attachment factor (46–50). However, the use of HS is most common for laboratory-adapted alphavirus strains. It has been observed that adaptation to the use of HS can be rapid, sometimes occurring in only a few passages in cultured cells. While this leads to increased cell culture infectivity, it generally decreases the pathogenicity of viruses *in vivo*. For example, CHIKV harboring the E282R mutation in E2, which increases HS interaction, exhibited reduced incidence of musculoskeletal disease in mice (51). SFV with lysine at E2 162 or 247 is more reliant on HS to enter cells, and upon peripheral (intraperitoneal or intravenous) infection, it appears to be attenuated. However, in intracranial infection, these mutant viruses

replicate to higher titers in the brain (52). In EEEV, mutagenesis of three conserved lysine residues in E2 abrogated binding to HS and decreased neurovirulence in mice (47). Thus, it is likely that for EEEV, high HS binding reduces viremia (thus negatively impacting the virus) but is compensated for by increased neurovirulence. However, additional studies are needed to reveal the role of HS in alphavirus infection in hosts and vectors. As a further complication, GAG is not the only molecule that acts as attachment factor for alphaviruses. The T-cell immunoglobulin mucin (TIM) domain family of proteins has been proposed to act as attachment factors for SINV, CHIKV, RRV, and EEEV (53). C-type lectins, including dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and liver-specific SIGN (L-SIGN), can also function as attachment factors for SINV. The expression of DC-SIGN or L-SIGN increased SINV binding and infectivity in human monocytic and primary dendritic cells (54). Previous studies suggested that heat shock cognate 70 protein (HSC70) and ATP synthase β (ATPSβ) are important for CHIKV entry into mosquito cells (55,55); however, a recent study suggested that the production of CHIKV in mosquito cells does not rely on these two attachment factors (56). This finding indicates that the attachment factor CHIKV uses to entry mosquito cells remains to be identified. Furthermore, alphaviruses may use different attachment factors in mammalian and mosquito cells; for instance, SINV binding to lectins was observed only in mosquito cells, which produced high-mannose or paucimannose N-linked glycans instead of the complex N-linked glycans in mammalian cells (the differences in glycans are due to protein posttranslational modifications in the cells of these two different organisms) (57). In summary, there are still many unanswered questions about the identities and roles of attachment factors in alphavirus entry.

Despite the importance of attachment factors in facilitating the contact of virions with host cells, it is the receptors that specifically bind to the E2 or E1 glycoprotein of the virion that allow their subsequent internalization. There have been multiple hurdles in identifying the *bona fide* receptors of alphaviruses, partly because of the lack of discernible interactions between putative receptors and purified E2 glycoproteins (58). This may be because many alphavirus receptors contact E1 in the E1/E2 dimer. In addition, alphaviruses can infect cells lacking one or more entry receptors because alphaviruses tend to use multiple receptors, and a lack of one (or even several) of them hampers, but does not completely prevent, virus infection. To complicate this situation even further, alphaviruses may use different receptors to bind to mosquito and mammalian cells, and as recently reported, in some cases, alphaviruses can bind to a receptor of one vertebrate species but not to its homolog from other species (59). Finally, the use of receptors differs among different alphaviruses, complicating the identification of receptors. Despite the complexity of discovering alphavirus receptors, however, much progress has been made recently, and several receptors have been identified. Natural resistance-associated macrophage protein (NRAMP) has been identified as a receptor for SINV in both insect and mammalian cells (60); however, in bird cells, SINV binds to matrix remodeling-associated protein 8 (Mxra 8) (59).

Mxra8 is the main receptor for numerous arthritogenic alphaviruses, including CHIKV, RRV, MAYV, and ONNV (61). Low-density lipoprotein receptor class A domain-containing 3 (LDLRAD3) was identified as a receptor for VEEV (62). Very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) have been reported to be receptors for SFV, SINV, and EEEV (63). Both Mxra 8 and LDLRAD3 bind to clefts formed by neighboring E1-E2 heterodimers (64–66). However, VLDLR, a receptor of SFV, binds to multiple E1-DIII sites through its LA repeats (67). This finding contrasts with the textbook knowledge that typically marks E2 as an antireceptor (i.e., a receptor-binding molecule) of alphaviruses. The list of proteins that function as receptors (or coreceptors) of alphaviruses is expected to expand, improving the understanding of alphavirus attachment to host cells.

The binding of the E2/E1 dimer to the receptor(s) triggers virion internalization. This process occurs via clathrin-mediated endocytosis and results in the delivery of virions to the endosomal compartment, where the viral envelope fuses with the endosomal membrane (68,69). At the low pH in endosomes, E2 dissociates from E1, which exposes the fusion peptide in E1 (initially blocked by E2). Subsequently, E1 forms a homotrimer that triggers membrane fusion and releases the nucleocapsid into the cytosol (70–72). Once the nucleocapsid has been released into the cytoplasm, the nucleocapsid must disassemble to expose G RNA for translation (Figure 5). However, this process is not well understood or well studied. However, it is known that the uncoating of nucleocapsids relies on their interaction with 60S ribosomal RNA (73,74), so this process is mediated by cellular ribosomes.

Once G RNA is released into the cytoplasm, it serves as mRNA for the translation of two nonstructural polyproteins, P123 (~90%) and P1234 (~10%). The synthesis of P1234 requires ribosome readthrough of the opal stop codon at the region encoding the carboxy terminus of nsP3 in the majority of alphavirus species (for example, SINV, most CHIKV strains) (75). However, some natural isolates of alphaviruses synthesize only P1234 because they lack the opal stop codon (76). The role of the opal stop codon has not been well studied; it has been shown that the presence of the opal stop codon in ONNV can increase its infectivity in mosquitoes (77). On the other hand, the infectivity of CHIKV in mammalian cells increased when the opal stop codon was replaced with an arginine codon (78). It is possible that the two variants (with and without the opal stop codon) coexist and dominate differently in mammalian (P1234) and insect cells (P123).

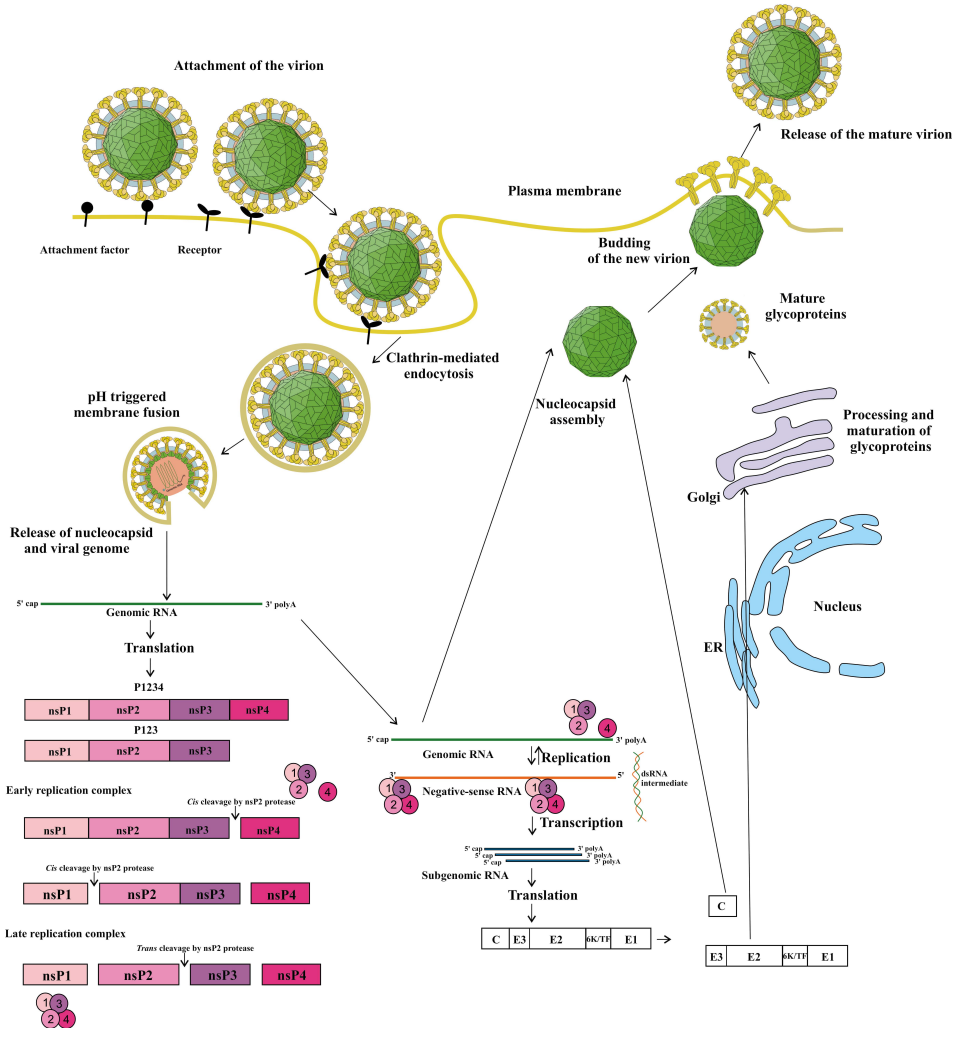

Figure 5. Overview of the alphavirus infection cycle. Alphavirus infection is initiated by the binding of virions to attachment factors and specific cellular receptors. The bound virions then enter cells by clathrin-dependent endocytosis. At low pH, the virion envelope fuses with endosomal membranes, releasing the nucleocapsid into the cytoplasm. Subsequently, G RNA is uncoated and translated, which results in the expression of the P123 or P1234 polyproteins. P1234 is converted into a replicase through an orchestrated cascade of proteolytic processing. The early replicase (first processing product) synthesizes negative-strand RNA, forming a dsRNA intermediate with G RNA. This dsRNA molecule is used by the late replicase (final processing product) to produce new G and SG RNAs. SG RNA acts as a mRNA to express structural polyproteins. The capsid protein is autocleaved and released into the cytoplasm, where it interacts with G RNAs to assemble into nucleocapsids. Glycoproteins are processed and matured in the ER and Golgi compartments and transported to the plasma membrane. The nucleocapsid interacts with glycoproteins to form virions, which are then released by budding. The virion is adapted from https://viralzone.expasy.org/625.

Synthesized nonstructural polyproteins are sequentially processed into individual nsPs by nsP2 protease activity (79). To form a functional RNA replicase, P1234 must be cleaved by the nsP2 protease at the site between nsP3 and nsP4 (3/4 site; similar naming is used hereafter for the cleavage sites between nsP1 and nsP2 (1/2 site) and between nsP2 and nsP3 (2/3 site)). This cleavage results in the formation of a short-lived early replicase complex (P123+nsP4) that synthesizes negative-strand RNA. The synthesis of negative-strand RNA is associated with spherule (membrane invagination) formation. Each G RNA is used as a template for the synthesis of a single negative-strand RNA, resulting in the formation of double-stranded (ds) RNA inside the spherule. Within 4–6 hours post-infection (h.p.i.), depending on the virus and the multiplicity of infection, the synthesis of negative-strand RNA is halted, and no new replicase complexes are formed. This is partially due to the conversion of P123+nsP4 to nsP1+P23+nsP4 by the nsP2 protease cleaving the 1/2 site *in cis*. A further processed replicase complex (nsP1+P23+nsP4) can synthesize G RNA and SG RNA; however, this complex likely does not contribute significantly to viral RNA synthesis because it is very short-lived. The final cleavage between nsP2 and nsP3 occurs rapidly *in trans,* forming a stable late replicase complex (nsP1+nsP2+nsP3+nsP4). The late replicase complex uses the negative strand of dsRNA as a template to synthesize large amounts of G RNAs and SG RNAs (Figure 5). Thus, the processing of P1234 by nsP2 protease activity is finely orchestrated and follows strict rules: *cis* cleavage at the 3/4 site must occur first, followed by *cis* cleavage at the 1/2 site, and finally , rapid cleavage, exclusively *in trans*, at the 2/3 site (80). Interestingly, however, the formation of a late replicase complex is not an absolute requirement for alphavirus replication. It has been shown for SINV and SFV that blocking cleavage at the 2/3 site alone or in combination with the 1/2 site is not fatal; mutant viruses can even generate more G RNA (if only the 2/3 site is blocked) and produce viable progeny (81,82). While these viruses replicate reasonably well in interferon-negative cells, they cannot replicate in interferon-positive cells. This is, at least mostly, because the nsP2 produced by these viruses cannot be released from nsP3 (or from nsP1 and nsP3) due to the blockage of cleavage sites. Therefore, it cannot enter the nucleus or inhibit cellular transcription, hampering its ability to interfere with the host's innate immune response. Another reason for the cessation of negative strand RNA synthesis is that, at the later stages of infection, free nsP2 accumulates in the cytoplasm of infected cells. This protein cleaves P1234 at the 2/3 site and produces P12 and P34, which cannot form a functional replicase complex. Instead, their processing (P12 is cleaved *in cis,* and P34 is cleaved *in trans*) results in the formation of nsPs that are not bound to replicase complexes. nsP1 is translocated to the plasma membrane, nsP2 is translocated mainly to the nucleus, nsP3 forms cytoplasmic aggregates, and nsP4 is mostly degraded by proteasomes (80,83).

The RNA replication of alphavirus occurs inside bulb-like spherules. The spherules are membrane invaginations with a diameter of approximately 50 nm; in fact, their size is not fixed but is regulated by the length of the RNA (dsRNA) located inside a spherule (84). The formation of dsRNA by the alphavirus early

replicase (P123+nsP4) is a prerequisite for spherule formation (85). In the early stages of alphavirus infection, spherules are formed and remain located on the plasma membrane; the interior of a spherule is connected to the cytoplasm via a narrow neck structure (85,86) (Figure 6). The neck region of the spherule consists of a dodecameric ring formed by nsP1, which has affinity for the plasma membrane (87). It has been assumed that the alphavirus RNA replicase must be located at the neck of the spherule (85,88); however, the architecture of the replication complex remained elusive until it was recently characterized by two groups. Taken together, these studies demonstrated that one molecule of nsP4 sits within the central pore of the dodecameric ring formed by nsP1; a single molecule of nsP2 is located at the top of nsP4 and interacts with it via the N-terminal helicase part of nsP2 (nsP2h). There is additional density above nsP2h, which is likely the nsP2 protease. This region of nsP2 is confined by a cytoplasmic ring that most likely consists of nsP3 and host factors, which interact with nsP3 (89,90) (Figure 6). At late stages post-infection with some alphaviruses (for example, SFV and RRV), spherules are internalized and localized on the internal surface of modified endosomes and lysosomes, forming so-called cytopathic vacuoles (CPV-1) (86). However, for CHIKV, the spherules remain mostly at the plasma membrane. This difference is due to the region of SFV nsP3, which can hyperactivate the phosphatidylinositol-3-kinase-Akt signaling pathway and, by unknown mechanisms, drive the internalization of spherules (91). Surprisingly, the lack of spherule internalization has no detectable impact on viral RNA synthesis (85); therefore, its importance for viruses remains unknown.

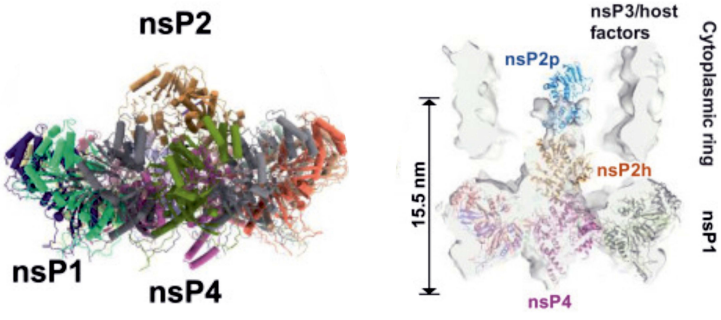

Figure 6. Graphical illustration of the architecture of the alphavirus replicase complex core (CHIKV nsP1+ CHIKV nsP2+ONNV nsP4). Left: Side view of the molecular structure, where 12 subunits of CHIKV nsP1 form the dodecameric ring. One subunit of ONNV nsP4 sits within the pore of the nsP1 ring, and the helicase part of nsP2 interacts with nsP4. Right: Model of the replicase complex based on cryo-EM images of spherules. Figure is adapted from (90).

1.2.2. Alphavirus virion assembly and budding

The structural polyprotein CP-E3-E2-6K-E1 or its variant CP-E3-E2-TF, synthesized due to a ribosomal frame shift in 6K, is translated from SG RNA (2,92). CP has a C-terminal serine protease domain and is autoproteolytically released into the cytoplasm (93). After the removal of CP, the membrane location signal in E3 is exposed, and consequently, the rest of the structural polyproteins are translocated to the endoplasmic reticulum (ER). In the ER, the remaining polyprotein is further processed by cellular enzymes into P62 (a precursor to the E3 and E2 proteins prior to cleavage with furin), 6K, and E1 (or P62 and TF if there is a frame shift during the translation of 6K). P62 interacts with E1 and forms heterodimers, which are subsequently transported to the *trans*-Golgi network, where P62 is cleaved by furin, resulting in the expression of individual E3 and E2 glycoproteins (94,95). The cleavage of P62 is required for formed virions to acquire the ability to enter and fuse with new cells. However, even after E3 is cleaved from E2, E3 remains in association with E2-E1 heterodimers in a pH-dependent manner. E3 has been shown to stabilize spikes to prevent premature fusion with cellular membranes under acidic conditions (96,97). However, in many alphaviruses, E3 dissociates from mature virions after budding, while for some alphaviruses, such as SFV, E3 remains associated with E2-E1 spikes (98). In this manner, E2-E1 heterodimers or E3-E2-E1 heterotrimers form trimers, making up 80 mature viral spikes.

During nucleocapsid assembly, CP recognizes a specific packaging signal (PS) in G RNA that directs encapsidation of the G RNA. Weiss and colleagues found that the SINV CP could bind nonviral RNA when its PS was included in these RNA molecules (99). The SINV PS sequence was found to interact with the N-terminal domain of CP (100). In most alphaviruses, the PS is located in the region encoding the ns-polyprotein; for example, the PS of SINV and VEEV is located in the nsP1 region (99,101), while for viruses belonging to the SFV clade, the PS is in the nsP2 region (102–104). Although the PS of SFV clade viruses is located in a different region, their capsid proteins can also use the PS of other alphaviruses, which are located in the nsP1 region. However, this is not reciprocal, so it is likely that the PS of SFV clade viruses evolved separately. It has been hypothesized that the PS in nsP1 was present in ancestral alphaviruses but was replaced by the PS located in nsP2 when the SFV clade emerged (101). The alphavirus NC assembly is a multistep process initiated by dimeric CP interacting with G RNA via its positively charged N-terminal domain (105). However, the mechanisms of alphavirus NC assembly have not been elucidated. It has been shown that NC assembly is not a process mediated only by a CP-PS interaction. The elements of the replication complex also play a role in packaging G RNA into NC. For instance, the expression of nsP1-3 in the form of the P123 precursor increased the ability of VEEV defective interfering (DI) RNAs to be packaged into virion-like particles (106,107), and there is evidence that nsP2 participates, possibly even directly, in alphavirus genome packaging (106).

The final stage of the alphavirus infection cycle is virion formation and budding. In vertebrate cells, this occurs on the plasma membrane. The preassembled NC (which has T4 icosahedral symmetry) is transported to the plasma membrane, where it interacts with the carboxy-terminal domain of E2. This allows the forming virion to acquire glycoprotein spikes (E2-E1 heterodimers or E3-E2-E1 heterotrimers) (108,109). During this process, the lipid bilayer of the membrane that encloses the NC is obtained. The transportation of glycoprotein spikes to the plasma membrane is promoted by 6K, a glycoprotein with ion channel activity (110). In addition to their role in transporting spikes, 6K and TF are also required for virus assembly and budding, possibly through interactions with E1 and E2 (111,112). Once the virions are released, they are ready to bind and enter susceptible and permissive cells to initiate a new infection cycle.

1.3. General components of the alphavirus replication complex

1.3.1. nsP1

nsP1 (~60 kDa) is the sole membrane-anchored nonstructural protein that is responsible for the localization of replication complexes to the plasma membrane. The cryo-EM structure of CHIKV nsP1 was revealed in 2021 by two groups (113,114). In transfected or infected cells, nsP1 forms ring structures. The ring consists of 12 subunits of nsP1 with C12 symmetry and can be divided into three regions: the crown, the waist and the skirt (Figure 7). In the complex, nsP1 folds into two domains: the capping domain (located at the top of the crown) and the membrane binding and oligomerization domain, which defines the waist and skirt regions of the complex. The crown has a negatively charged, cone-shaped inner chamber with a 14 nm diameter at the top, which narrows to 7 nm in the waist region, forming a pore 2 nm in depth.

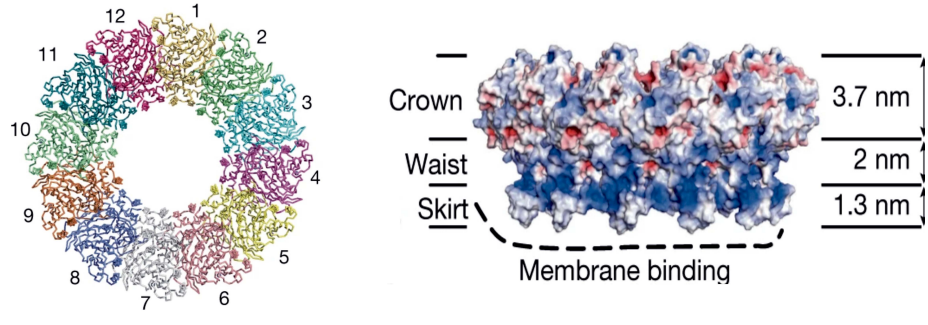

Figure 7. Cryo-EM structure of the spherule-pore complex of CHIKV nsP1. Left: Cytosolic view of the nsP1 complex formed with 12-fold symmetry. Right: Lateral view of the nsP1 complex. Figure is adapted from (113) with permission from Springer Nature.

This pore is large enough to allow the transit of RNA, nucleotides, and globular proteins (smaller than 70–90 kDa); however, in the viral replicase complex, the pore is not empty but is occupied by the nsP4 molecule (Figure 6). Under the skirt, there are membrane-binding spikes that project rigidly from the complex and are positively charged, attracting the membrane phospholipid heads and possibly inducing the membrane bending that is observed in the spherule necks.

Moreover, nsP1 has N7-guanine-methyltransferase (MTase) and guanylyltransferase (GTase) activities that are required for the addition of cap 0 (7-methyl-GpppA, covalent linkage of an m^7GMP moiety to the first nucleoside of the RNA via a 5'-5' triphosphate bond) structures to viral positive-strand RNAs (115,116). Point mutations that abolish MTase/GTase activities render the virus noninfectious, implying an important role for nsP1 in viral RNA capping (117). The capping of alphavirus RNAs is performed in three steps. During the first step, the RNA triphosphatase activity of nsP2 removes 5′ γ-phosphate from the RNA. Next, a methyl group from S-adenosyl methionine (AdoMet) is transferred to GTP by the MTase activity of nsP1, which yields m^7GTP. Afterward, m^7GTP forms a covalent bond with the conserved histidine residue of nsP1, which is subsequently transferred to the 5' end of RNA via the GTPase activity of nsP1 (115). The capping steps are as follows:

1) pppRNA → ppRNA + P_I

2) GTP + AdoMet → m^7GTP + AdoHcy

3) m^7GTP + nsP1 → m^7GMP-nsP1 + ppRNA → m^7GpppRNA

The first two highly conserved nucleotides (AU) in G RNAs are required for nsP1 to cap the viral RNA. RNA of the same size with a GAG sequence at the 5' end (instead of native AUG) cannot be capped by CHIKV nsP1 (118). For alphaviruses, RNA capping occurs before stem loop structures at the 5' end are formed, most likely when the RNA is still very short. While RNA capping is required for alphavirus infectivity, it is not absolute; i.e., not all synthesized G RNAs are capped. The increased capping activity of SINV nsP1 decreases infection, revealing that finely tuned capping activity is important for viral fitness (119). The noncapped G RNAs are packaged into virions and may play a role in activating host innate immunity, which is correlated with the type 1 IFN response *in vitro*. (120). Several studies have revealed that nsP1 can also decap RNA substrates, including cellular mRNAs (118).

nsP1 contains an amphipathic helix formed by a conserved short peptide (G_{245}STLYTESRKLLRSWHLPSV$_{264}$), which binds to the membrane (87). This amphipathic peptide segment is crucial for membrane association, as it participates in forming the membrane-association helix, as shown in the CHIKV nsP1 cryo-EM structure by Zhang and colleagues (114). It is also essential for alphavirus RNA replication. Spuul and colleagues showed that the R253E mutation, introduced into the amphipathic peptide of nsP1 of SFV, drastically affects nsP1

localization, causing it to localize to the cytoplasm instead of the plasma membrane; this mutation was found to be lethal in the context of the virus genome (121), and the same was observed for analogous mutation in CHIKV nsP1 (122). The membrane binding affinity of alphavirus nsP1 is increased by palmitoylation. CHIKV and SFV nsP1 contain three cysteine residues (^{418}CCC^{420}), but SINV nsP1 contains one cysteine residue (C^{420}) (123). Although nsP1 palmitoylation is conserved in alphaviruses, the effects of mutations in the palmitoylation sites are different. A lack of nsP1 palmitoylation in CHIKV completely abolishes its replication because CHIKV nsP1 palmitoylation is critical for targeting the replicase complex to cholesterol-rich plasma membrane domains, which is important for CHIKV replication (124). However, SINV and SFV with palmitoylation-defective nsP1 are viable. In BHK cells, the growth rate of palmitoylation-defective SINV was similar to that of wild-type SINV, while slower growth was observed for the SFV mutant. There was also a major change in the SFV phenotype *in vivo*: no neurovirulence was observed in mice infected with the mutant virus. In addition, the subcellular localization of nonpalmitoylated nsP1 differed from that of its wild-type counterpart: the mutant protein showed diffuse staining in the cytoplasm instead of being exclusively present on the plasma membrane (characteristic of wild-type nsP1 in SFV). However, both the SFV and SINV palmitoylation mutants presented CPV-1 structures similar to those of wild-type viruses, indicating that the membrane affinity of the SINV and SFV replication complex is independent of the palmitoylation of nsP1 (123). However, the data on palmitoylation-defective SFV were later found to be flawed. Specifically, another study showed that the survival of the SFV palmitoylation mutant is due to adaptive mutations in nsP1. These mutations did not restore nsP1 palmitoylation, but they restored the interaction between nsP1 and nsP4 that was disrupted by replacement of the three palmitoylated cysteines with alanine residues (125). This finding indicates that the functional interaction between nsP1 and nsP4 is essential for alphavirus replication. Indeed, functional interactions between nsP1 and nsP4 have also been observed in other studies. For example, there is a SINV temperature-sensitive (ts) double mutant in which mutations in both nsP1 and nsP4 are necessary for the ts phenotype. The interaction between nsP1 and nsP4, as shown in Figure 6, was clearly confirmed by cryo-EM analysis of the structure of the replicase complex (89,90).

The interaction between nsP1 and nsP4 implies that nsP1 has a role in RNA replication. These experiments suggested that nsP1 may play a leading role in negative-strand synthesis. Sawicki's group reported that a mutation in the nsP1 gene of SINV (ts11, A348T) could cause a cease in negative-strand RNA synthesis at a nonpermissive temperature (40 °C). The nsP4 mutant of SINV allowed negative-strand synthesis to proceed at 40 °C. However, when these two mutations were combined into the same SINV genome, negative-strand synthesis was not restored. Thus, with respect to negative-strand RNA synthesis, the mutation in nsP1 dominated the mutation in the nsP4 protein (126). Another study by the Strauss group revealed that a mutation in nsP1 (T349K) could allow viruses harboring

nsP4 with an N-terminal Ala, Arg, or Leu residue (instead of native Tyr) to replicate and produce progeny, albeit in a temperature-sensitive manner; in the absence of the compensatory mutation in nsP1, those mutants were nonviable (127). These studies suggest that nsP1 (probably residues A348-T349 in polyprotein P123) interacts with the N-terminus of nsP4 and that this interaction is required for the recognition of the promoter used for the initiation of negative-strand RNA synthesis.

In addition to its role in the replication complex, nsP1 has other functions. For example, nsP1 was identified as a critical determinant of the pathogenesis of RRV-induced musculoskeletal inflammatory disease in mice (128). Later, monocytes were found to be critical for controlling acute alphavirus infection and reducing the severity of disease in mice by increasing the expression level of type 1 IFN. However, this response can be counteracted by nsP1 (129). In one study, palmitoylation-defective SFV did not cause neurovirulence in infected mice, suggesting that for SFV, the region that determines pathogenesis overlaps with the palmitoylation sites of nsP1 (123). It should, however, be noted that some of these effects may actually be related to the role of nsP1 in RNA replication: the induction of type I IFN is related to the efficiency of synthesis of IFN-inducing RNAs (130), and palmitoylation-defective SFV was not viable in the absence of compensatory changes (125).

Another intriguing function of nsP1 is its ability to remodel cellular membranes in infected cells or even when it is expressed alone. In cells expressing nsP1 from SINV, SFV, or CHIKV, nsP1 induces the formation of filopodia-like extensions; this effect depends on the palmitoylation of nsP1 (123–125). *Bona fide* filopodia are thin membrane protrusions with a diameter of 100–200 nm that can reach up to 10 μm or greater in length. These extensions are important for sensing the extracellular environment and may be important for the cell-to-cell transmission of viral infections. To induce the formation of filopodia-like structures, nsP1 requires the active form of Rac1, a protein in the Rho family of GTPases (a family of small ~21 kDa signaling G proteins that sense and mediate extracellular signals) (124). Interestingly, filopodia-like structures were not observed when the P123 polyprotein and nsP4 were coexpressed in cells, although these cells form structures similar to the spherules observed during viral infection (131).

1.3.2. nsP2

nsP2 (~90 kDa) is the largest nonstructural protein of alphaviruses. nsP2 consists of an N-terminal RNA helicase region (nsP2h) and a C-terminal protease region (nsP2p). These two regions are connected by a short flexible linker (residues 463–476) (132) (Figure 8). nsP2h consists of multiple domains/subdomains, including the N-terminal domain (NTD), stalk, superfamily 1 accessory domain 1B, and helicase core (two similar Rec-A-like domains, RecA1 and RecA2) (133). nsP2p consists of two domains, a papain-like cysteine protease domain and an S-adenosyl-L-methionine (SAM)-dependent RNA methyltransferase-like (MTL) domain. nsP2 is a multifunctional protein with four known enzymatic activities and several important nonenzymatic activities.

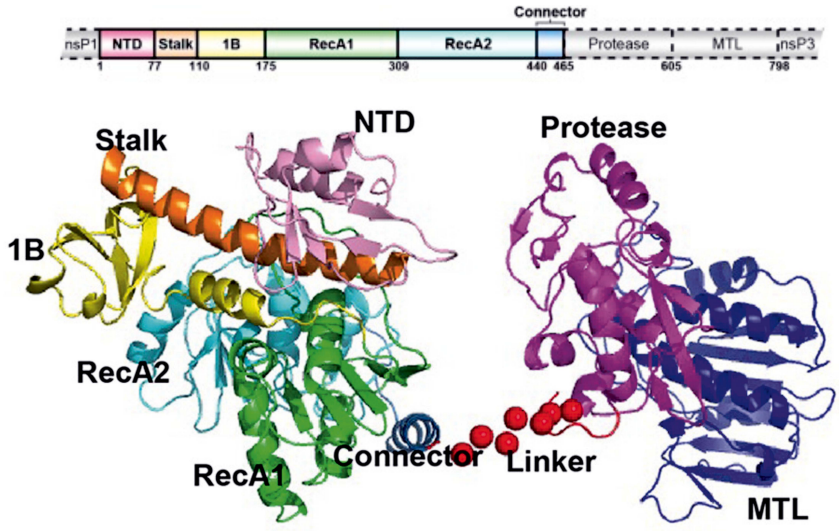

Figure 8. Overview of the organization and 3D structure of CHIKV nsP2. CHIKV nsP2h and nsP2p are connected via a flexible linker. NTD: N-terminal domain of nsP2; MTL: S-adenosyl-L-methionine (SAM)-dependent RNA methyltransferase-like domain. The figure is adapted from (132).

nsP2h has nucleoside triphosphatase (NTPase) and RNA 5′ γ-phosphatase (RTPase) activities and has been shown to bind RNA at the top of the groove covered by the N-terminal domain of nsP2 (NTD, stalk and 1B). nsP2h contains three hydrophobic residues that form stacking interactions with RNA (133). As the structure and its interactions with an RNA template were first described in Publication I, a more detailed description of nsP2h is provided in the Results and Discussion section. Interestingly, nsP2h alone has no detectable RNA helicase activity; this activity is only observed for full-length nsP2, i.e., the presence of the nsP2p region is required. RNA helicases are motor proteins that use the energy produced by the hydrolysis of NTPs to unwind dsRNA. The alphavirus nsP2 helicase is categorized into superfamily 1 based on conserved Rec-A-like domains (134). Based on the sequence, the 470 N-terminal residues should have helicase activity; however, as stated above, only full-length nsP2 can unwind dsRNA (135). Furthermore, nsP2 has RNA annealing activity that also depends on the presence of the C-terminal domain of nsP2p. Although the nsP2h region alone has NTPase activity, this activity appears to be attenuated; in the presence of the C-terminal domain of nsP2 (i.e., in the full-length nsP2), the NTPase activity was 5–7 times greater than that for nsP2h alone (135). Moreover, studies have shown that NTPase activity is necessary for the RNA helicase and RTPase activities of nsP2, as mutations that inhibit NTPase activity result in the loss of these activities as well (136,137); most likely, all three activities depend on the same active site. The NTPase activity of nsP2h contributes to the induction of host cell translational shut-off through promoting the phosphorylation of eukaryotic elongation factor 2 (eEF2) (138). The RTPase activity of nsP2 is responsible for the removal of

γ-phosphate from the 5' end of positive-sense alphavirus RNAs to yield G and SG RNAs with 5' diphosphate structures, which are used as substrates for nsP1-mediated capping reactions (136).

The protease activity of nsP2p is responsible for orchestrating the processing of the nonstructural polyproteins. As described above (1.2.1), during the formation of the active RNA replicase, the 3/4 site is processed first, followed by *cis* cleavage of the 1/2 site, whereas the final cleavage—that of the 2/3 site—occurs *in trans* (79). This function is essential for viral replication, and its protease activity has been shown to be modulated by the nsP2h region and other parts of nsP2 containing polyproteins (139). For example, the proteolytic activities of the SFV, SINV, and CHIKV nsP2 proteases also depend on the NTD of nsP2 and the macro domain of nsP3 (140,141). nsP2h is proposed to function as a cofactor of the protease, similar to the flavivirus NS2B protein (142). The nsP2p-mediated processing of P1234 into P123 and nsP4 is an absolute requirement for alphavirus replication (143). In contrast, P123 processing is not absolutely required, and mutant viruses unable to cleave the 2/3 site or the 1/2 and 2/3 sites are able to replicate (82,144). The modes (*cis* or *trans*) of cleavage of the 3/4, 1/2, and 2/3 sites and the efficiencies of these cleavages are different as well. During early infection, the protease preferentially cleaves the 3/4 site *in cis*. This is likely because at this time, the concentration of nonstructural proteins in infected cells is low, and therefore, *cis*-cleavage is favored. The 1/2 site of SINV and SFV has been shown to be cleaved slowly *in cis* (80); however, there are old data indicating that at increased concentrations of P123, cleavage occurs mostly *in trans* (2), suggesting that processing of the 1/2 site is temporally regulated. Whether this is the case remains uncertain, as it has been clearly demonstrated that slow cleavage of the 1/2 site is crucial for alphaviruses and that viruses in which this cleavage is accelerated due to mutations in nsP2 and/or in the cleavage site are attenuated or not viable (145). Furthermore, unpublished data from our laboratory and from our collaborators clearly show that the speed of 1/2 site processing is decreased by the presence of RNA. All these findings are in line with the hypothesis that 1/2 site processing serves as a molecular trimer—this process is purposefully delayed to allow the transport of replicase precursors to the plasma membrane, initiation of the formation of spherules and, perhaps, the completion of negative-strand RNA synthesis. Once the 1/2 site is cleaved, the protease is ready for cleavage at the 2/3 site (83). Unlike the first two cleavages, this cleavage must occur *in trans*, as structural analysis of nsP2 and nsP3 has shown that the 2/3 site is too distant from the nsP2 protease active site to be cleaved by the same molecule (146). Cleavage at the 2/3 site is faster than that at the other sites, and the processing intermediate P23 is therefore difficult to detect. The high efficiency and *trans* mode of cleavage at the 2/3 site is used by viruses to prevent the formation of excessive replication complexes; once free nsP2 accumulates in infected cells, it can cleave newly translated nonstructural proteins, resulting in the formation of P12 and P34 (i.e., processing switches to the nonreplicative pathway).

In addition to its enzymatic activities, nsP2 functions in the initiation of SG RNA synthesis, as several ts mutations in the nsP2 helicase domain were shown

to influence SG RNA synthesis efficiency (147,148). Another well-documented property of nsP2 is its translocation to the nucleus and nucleolus of vertebrate cells (88,149). Old World alphavirus nsP2 proteins contain either one or two putative nuclear localization signals (NLSs), which are assumed to act as the main means of nsP2 accumulation in the nucleus. For SFV, mutations in the NLS affect nsP2 translocation into the nucleus (150); however, for the SINV (putative) NLS mutant, no effect on the nuclear localization of nsP2 was observed (151). CHIKV nsP2 lacks a clear NLS, and mutations in the region corresponding to the NLS in the nsP2 of SFV do not abolish the nuclear localization of CHIKV nsP2 (152). Thus, exactly how the nuclear transport of nsP2 occurs is not clear. Regardless of how the protein is transported to the nucleus, SFV, CHIKV, and SINV nsP2 proteins have been shown to inhibit host mRNA transcription by degrading the catalytic subunit of DNA-dependent RNA polymerase II (RPB1) (153). Interestingly, nsP2-induced transcriptional shutoff is not due to the protease activity of nsP2, as nsP2 lacking protease activity induces RPB1 degradation at a similar level to wt nsP2. In contrast, mutations in the helicase or SAM-MTase-like domain of nsP2, reported for noncytopathic replicons of alphaviruses, block the ability of nsP2 to induce proteasome-mediated degradation of RPB1 (153,154). Downregulation of the transcription of cellular mRNA is an efficient way to reduce the antiviral response, as it suppresses the expression of cellular interferon-inducible genes. The degradation of RPB1 occurs early after infection and is completed by 6 h.p.i., i.e., before other virus infection-induced changes (apoptosis, autophagy, and the inhibition of STAT1 phosphorylation) occur. Thus, the degradation of RPB1 induced by nsP2 is a key mechanism used by Old World alphaviruses to evade the innate immune response. However, RPB1 degradation is not the only mechanism by which nsP2 counteracts host cell responses. nsP2 of CHIKV can also inhibit the innate immune response by blocking STAT1 phosphorylation and thereby inhibiting the IFN-stimulated Janus kinase–signal transducer and activator of transcription pathway (JAK-STAT) (155,156). Furthermore, CHIKV nsP2 interacts with the human autophagy receptor NDP52. This interaction can restrict the localization of nsP2 to the nucleus and prolong CHIKV replication in human cells (157,158). Moreover, transiently expressed CHIKV nsP2 can inhibit the unfolded protein response (UPR). In infected cells, the UPR is activated due to the ER stress caused by the expression of alphavirus glycoproteins (159). The UPR has an antiviral effect, and viruses harboring noncytotoxic mutations in nsP2 (which abolish host shut-off) cannot interfere with the UPR (160). Thus, there are multiple mechanisms by which nsP2 interferes with antiviral responses in cells. It is plausible that the different mechanisms are used under different conditions, e.g., in different cell types or at different stages of infection.

1.3.3. nsP3

The alphavirus nsP3 has three domains: the macro domain, the alphavirus unique domain (AUD), and the hypervariable domain (Figure 9). It has long been clear that nsP3 is essential for viral RNA synthesis, as mutations in nsP3 have been shown to influence the initiation of minus-strand synthesis as well as SG RNA synthesis (161). Among all alphavirus nsPs, nsP3 has the least thoroughly elucidated functions. Nevertheless, much progress has been made in recent years.

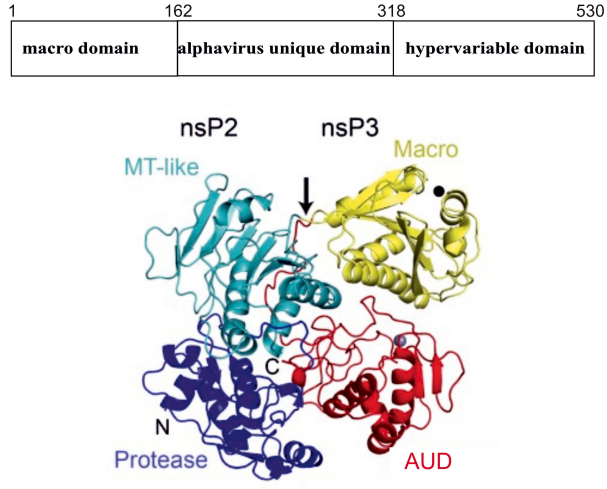

Figure 9. Overview of the structural organization of CHIKV nsP3 and the 3D structure of SINV nsP23$^{Pro\text{-}AUD}$. In the resolved structure of the nsP2 protease and the first two domains of nsP3, the 2/3 site (arrow) is located at the cleft formed by the MTL of nsP2 and the macro domain of nsP3. The black dot represents the ADP ribose-binding site. The figure is adapted from (146).

The macro domain of nsP3 is highly conserved among alphaviruses and is homologous to similar domains found in the nsPs of many RNA viruses (coronaviruses, rubella virus, and hepatitis E virus) and in some bacterial and cellular proteins (162). The macro domain functions in ADP-ribose (ADPr)-mediated signaling, can bind mono-ADPr (MAR) or poly-ADPr (PAR) as well as remove ADPr from MARylated substrates, and can diphosphate the ADP-ribose-1"-phosphate (163, 164). The ADP-ribosylhydrose activity of the macro domain probably has many functions. It can remove the ADPr modification from G3BP1 (Ras GAP-SH3 domain-binding protein), which is necessary for the disassembly of virus-induced stress granules (165). Possibly more importantly, the MAR hydrolase activity of the macro domain can remove ADPr from nsP2. It has been shown that this modification suppresses the activity of nsP2, and thus, MAR hydrolase activity serves to reactivate the protease activity of nsP2; this is one of the reasons why the macro domain is needed for nsP2 to cleave the 2/3 site (141,166). CHIKV mutants that cannot remove ADPr from MARylated proteins replicate slowly in mammalian neuronal cells and exhibit reduced virulence in 2-day-old mice (164). For SINV and VEEV, a mutation in amino acid residue 31 of nsP3 can compensate for the

negative effects caused by other mutations in nsP3 (for SINV mutations at amino acid residue 10, for VEEV, the HVD can be replaced with GFP) (167,168). Clearly, mutations in the macro domain have multiple effects that may be different for different alphaviruses. Finally, the macro domain is essential for nsP3 phosphorylation, as a mutation in the macro domain of nsP3 of SINV prevents its phosphorylation and affects negative-strand RNA synthesis (169).

The macro domain is followed by the AUD, which has four conserved cysteine residues for binding Zn^{2+} ions; these conserved cysteine residues are essential for SINV replication (146). In addition, there is little information about the functions of AUD, and its role in alphavirus replication remains unclear. For CHIKV and VEEV, it has been shown that AUD does acquire compensatory mutations that can restore the deleterious effects caused by HVD mutations (167,170). Thus, it is highly likely that AUD functions in combination with the macro domain and HVD. One study has shown that the AUD is essential for CHIKV replication, as mutants in this region exhibit a dramatic reduction in the production of infectious viruses due to a block in SG RNA (171). The AUD also contributes to the neurovirulence of SFV (172). This available information is, at best, loosely connected. Clearly, additional research is needed to fully elucidate the functions of AUD and its functional interactions with other domains of nsP3.

Unlike the macro domain and AUD, the HVD is intrinsically disordered. As its name implies, its length varies among alphaviruses, and its sequence similarity is also low. The HVD can tolerate the insertion of large protein sequences, such as mCherry, GFP and luciferases, without major impacts on viral replication (173–175). Furthermore, VEEV is viable even if the entire HVD is deleted (176). However, this does not mean that the HVD has no importance or no functions. HVD is heavily phosphorylated at serine and threonine residues (177). Due to the lack of conservation in the HVD, there are large variations in the phosphorylation sites of HVD from different alphavirus species (177,178), as well as in the impact of this modification. In SFV, preventing HVD phosphorylation has only mild effects on viral growth kinetics *in vitro*; this effect is more readily detectable *in vivo,* as mutant viruses have reduced pathogenicity in mice (177). In contrast, the CHIKV phosphorylation-negative mutant is noninfectious due to a lack of replicase RNA activity (178). The phosphorylation of the HVD is important for the replication of VEEV in mosquito cells but not in vertebrate cells (167).

Like other intrinsically disordered domains, the HVD serves as a hub for interactions with host proteins. In infected cells, nsP3 can form different cytoplasmic complexes—replication complexes and aggregates—that have no association with dsRNA or membrane components. For Old World alphaviruses, such as SINV, both nsP3-containing complexes are characterized by the presence of high levels of G3BP1 and G3BP2, which are components of cellular stress granules (179). The interaction of G3BPs with nsP3 is mediated by the NTF2-like domain of G3BPs and short peptide sequences present in the HVD (179,180). This interaction prevents the formation of *bona fide* stress granules. In addition, G3BPs have a proviral function, as the depletion of these proteins blocks CHIKV replication; this defect occurs at the stage of negative-strand RNA synthesis (181).

The interaction of HVD with G3BPs is important for Old World alphaviruses, although to different extents. For CHIKV, this interaction is absolutely essential, but for other Old World alphaviruses, depletion of G3BPs reduces replication (by only 1–3 orders of magnitude) but does not block replication entirely. The effects of G3BPs depletion in Old World alphaviruses depend on the Arg residue at the P4 position of the 1/2 site (181), suggesting that the functions of G3BPs are connected to replicase polyprotein processing and/or the formation of early replicase complexes. For New World alphaviruses, such as VEEV, the HVD binds to FXR proteins instead of G3BPs; again, this interaction is essential for virus replication. Interestingly, the HVD of EEEV can bind to both G3BP and FXR proteins, and the deletion of either of these proteins (but not both) has no effect on its replication (173,174). Overall, the interaction of HVD with G3BPs (or its mosquito homologs called Rasputin or Rin) inhibits the formation of stress granules and is critical for the formation of active replication complexes. In addition to G3BPs, the HVD can interact with large sets of other host factors via other short linear motifs. Binding motifs for some of these proteins, including FHL1, CD2AP, NAPIL1/NAPIL4, SH3KBP1, and amphiphysins, have been mapped (182–185). Taken separately, each of these proteins has a relatively minor impact on viral replication. However, in experiments using CHIKV HVD (in which G3BP binding motifs were not altered), eliminating the binding sites of all these factors made the virus nonviable in both vertebrate and mosquito cells (170). Thus, these factors are collectively crucial for a virus, indicating that the binding of G3BP alone to HVD is not sufficient to achieve a detectable level of CHIKV replication. In addition to common host factors, some host proteins bind only to the nsP3 of specific alphaviruses. For example, WDR48 is detected only in complexes formed by the HVD of SINV, while NAPIL1 and NAPIL4 interact only with the HVD of CHIKV (174,182).

In addition to its multiple roles in replication, nsP3 contributes to the vector specificity of CHIKV and ONNV and to the neurovirulence of SFV (23,186). The nsP3 of SINV has been shown to be responsible for host cellular translation shutdown (187). Moreover, nsP3 participates in activating the phosphatidylinositol-3-kinase-Akt-mTOR signaling pathway, although the activation effect is greater for SFV than for CHIKV. This effect can be switched by swapping the HVD of these two viruses (91). Taken together, these findings suggest that this protein is involved in all aspects of alphavirus infection; however, in most cases, the exact mechanism(s) is unknown or poorly understood.

1.3.4. nsP4

In alphavirus-infected cells, nsP4 is the least abundant viral protein: compared to other nsPs, its amounts are about 100-fold lower levels. This is because in most alphaviruses, there is an opal stop codon located between the regions encoding nsP3 and nsP4, and readthrough occurs with 5–20% efficiency (75,188). Accordingly, substantially fewer nsP4 molecules will be expressed. Additionally, the

conserved N-terminal tyrosine of nsP4 is known as a destabilizing amino acid residue, and as a consequence, free nsP4 is targeted for degradation by the proteasome (189). Taken together, these findings clearly indicate that alphaviruses downregulate the expression of nsP4, presumably for a purpose. One reason is obvious: nsP4 is the catalytic subunit of the RNA-dependent RNA polymerase (RdRp) of alphaviruses.

nsP4 consists of two domains: the N-terminal domain (NTD), which is unique to alphaviruses, and the C-terminal domain, which is characteristic of the virus-encoded RdRp and contains a conserved "polymerase motif" (GDD sequence). An early study showed that the SINV temperature-sensitive mutant ts6, which has a Gly-to-Glu substitution at position 153 of nsP4, was completely unable to synthesize RNA at nonpermissive temperatures, providing the first functional confirmation that nsP4 is the virus RdRp (190). Recently, this finding was confirmed by X-ray crystallography, which revealed that the nsP4 of SINV and RRV have characteristic right-hand folds; that is, the structures of these enzymes are similar to those of other viral RdRps. These two nsP4 structures are the only available alphavirus nsP4 structures, since nsP4 is difficult to manipulate due to its low stability and solubility (191). The structure of RRV RdRp is shown in Figure 10. In this structure, the finger-palm-thumb subdomains are sequentially arranged from the N-terminus to the C-terminus, and the extended fingertips with the thumb form the RdRp-distinctive encircled ring conformation required for RNA polymerization. However, many residues cannot be seen in the structure, indicating that these residues are located in flexible regions. Furthermore, 101 residues belonging to the N-terminal and C-terminal domains of nsP4 were also not visible in the structure, indicating that the NTD and C-terminal tail of nsP4 are intrinsically disordered and have dynamic structures in solution. Considering that nsP4 is an unstable protein, these findings indicate that, at least in infected cells, nsP4 must be stabilized, most likely by other nsPs. This finding was directly confirmed by the recently defined structure of the alphavirus RNA replicase core (Figure 6): nsP4 does not function as an individual protein; instead, it forms a complex with nsP1 and nsP2. Interestingly, in this structure, both the N- and C-terminal domains of nsP4 are well folded, and the complex has markedly greater RdRp activity than does nsP4 alone (90).

Several other lines of evidence indicate that nsP4 is directly responsible for alphavirus RNA synthesis. When alphaviruses are cultured in the presence of a broad-spectrum viral polymerase inhibitor, resistance-associated mutations can be detected in nsP4. For example, passaging of CHIKV in the presence of ribavirin and 5-fluorouracil led to the Cys 483 to Tyr mutation in nsP4, which confers resistance to RNA mutagens and increases the replication fidelity of CHIKV. However, this mutation also reduces genetic diversity, negatively influencing CHIKV fitness in invertebrate vectors and vertebrate hosts (192). In another study, an increased mutation frequency was observed when the Cys 483 residue was changed to Trp, Ala, or Gly (193). Our collaborators performed a similar selection experiment with CHIKV in the presence of 4-fluorouracil. Again, the Cys 483 Tyr mutation was obtained; alternatively, another substitution

in nsP4 (Q192L), which was also responsible for 4-fluorouracil resistance, was identified. The latter substitution did not change the replication fidelity of CHIKV. This finding suggested that the Cys483 residue of nsP4 is not a classical determinant of alphavirus polymerase fidelity but is more relevant to the ability to replicate under nonoptimal conditions.

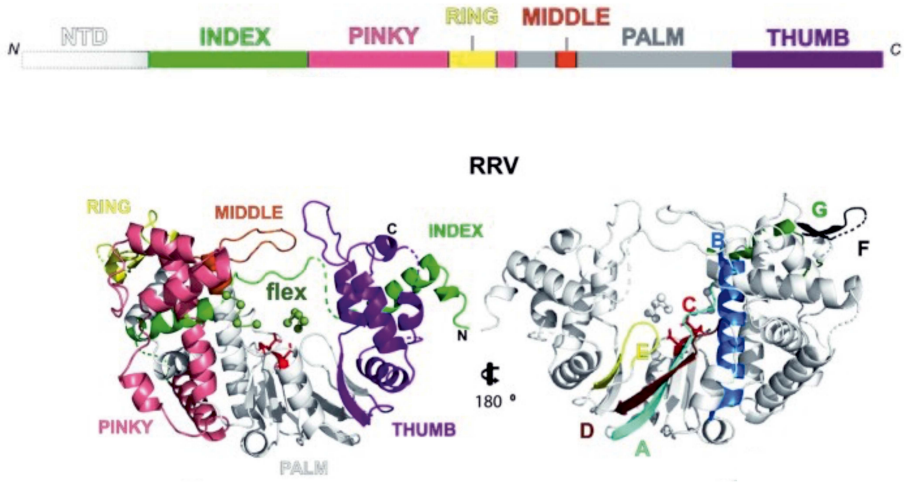

Figure 10. Overview of the organization and 3D structure of RRV-nsP4. The resolved structure of the RRV nsP4 has a right-hand fold with fingers (index, middle, right, ring and pinky), palm, and thumbs subdomains. Figure is adapted from (191).

The C-terminal RdRp domain of the alphavirus nsP4 possesses terminal adenylyltransferase activity (194), which can synthesize the poly (A) tails of alphavirus G and SG RNAs in a template-independent manner. This activity is needed because, in contrast to previous assumptions, the negative-strand RNA of alphaviruses lacks a poly(U) sequence. This is because its synthesis is initiated at the C-nucleotide located immediately before the poly(A) tail and not at the end of the poly(A) tail (195).

The conserved N-terminal tyrosine of the alphavirus nsP4 is essential for viral viability. For SINV, the tyrosine residue can be replaced only by another aromatic amino acid or histidine (196). Other substitutions decrease viral replication but can be compensated for by the addition of AU, AUU or AUA nucleotides to the 5’ end of the viral genome or by changing the third nucleotide in the viral genome from A to U. These data indicate that nsP4 interacts with the 5’ end of the viral genome (197). Considering the localization of the N-terminal Tyr residue in the structure of the replicase core and the fact that alphavirus proteins interact with nucleic acids through stacking interactions, it is reasonable to assume that the N-terminal Tyr residue (or other aromatic amino acid residues) interacts with the nucleobase of incoming nucleotides, possibly with the GTP that is used to initiate the synthesis of new RNA molecules. Thus, the N-terminal tyrosine may prime negative-strand RNA synthesis by forming a stacking interaction with GTP and G RNA. It has also been shown that SINV nsP4 specifically binds to the G and

SG promoter sequences in negative-strand RNA, and these interactions are mapped to different peptide fragments in nsP4 (198). Moreover, mutagenesis of SINV nsP4 revealed that the arginine 183 residue of nsP4 is important for negative-strand RNA synthesis and is functionally linked to the asparagine 374 residue of nsP1 (199,200). In addition, Rupp and colleagues showed that three mutations in the NTD of SINV nsP4 interfere with viral RNA synthesis, and this phenotype can be reverted by additional mutations in nsP1, nsP2, and nsP3 (201). In line with this observation, alphavirus nsP4 regions can be swapped, and swapping often results in functional replicases and viable viruses. This indicates that functional replicase cores can be formed from nsP1 and nsP2 of one alphavirus and nsP4 of another alphavirus. Due to the high conservation of nsP4, template RNAs of many alphaviruses can also be used by replicases of different alphaviruses (202).

Despite its low abundance and stability in alphavirus-infected cells, nsP4 has several functions, similar to other nsPs, that are not directly related to viral RNA synthesis. Thus, nsP4 also plays a role in antagonizing the UPR. As nsP2 has been reported to have a similar function, it is possible that these two proteins work together to counteract the UPR. Transient expression of an EGFP-nsP4 fusion protein is sufficient to prevent serine-51 phosphorylation of eukaryotic translation initiation factor (eIF2α); thus, nsP4 can counteract the antiviral effect of the PKR-like ER resident kinase (PERK) pathway, one of three downstream effector branches of the mammalian UPR (203). Additionally, CHIKV nsP4 interacts with the heat shock protein 90 alpha subunit (HSP-90α), and this interaction may be critical for viral replication (204).

1.3.5. Host factors

Multiple components encoded by host cells have been found to participate in the formation of replication complexes of alphaviruses. Often, these factors interact with viral RNA or nsPs and can therefore be coisolated with viral factors. For example, using mass spectrometry, a large number of cellular factors that interact with nsPs were found in cells infected with tagged CHIKV, SINV, and VEEV (176,205,206). Other methods used to identify host factors include siRNA knockdown, CRISPR–Cas9 knockout, yeast two-hybrid screens, magnetic pull-down of replication organelles and several others. Collectively, these efforts have identified hundreds of cellular proteins as potential host factors for alphaviruses. Somewhat surprisingly, different methods tend to result in sets of host factors with very little or no overlap between these sets. Host factors typically include RNA-binding proteins, translation machinery components, enzymes involved in lipid metabolism, and multiple components of the host innate immune response. This set is very diverse, as are the roles of these factors in alphavirus infection (although, in many cases, the roles are not known or not proven). However, very few host factors have been shown to function in viral RNA synthesis.

As described above, G3BPs interact with the nsP3 of Old World alphaviruses and with that of EEEV. These abundant vertebrate cell-specific proteins play critical roles in the replication of many alphaviruses; in their absence, viral negative-strand RNA synthesis is reduced or abolished (174,207,208). G3BPs bind to duplicated FGDF motifs in nsP3 via their N-terminal NTF2-like domain and interact with RNA via their C-terminal RNA recognition motif (RRM) and arginine- and glycine-rich RG/RGG regions (209,210). G3BPs are critical for the replication of CHIKV, RRV, and ONNV (181), which can be referred to as highly G3BPs sensitive alphaviruses. It has been shown that CHIKV cannot replicate at all in U2OS-derived double-null ΔΔ G3BP1/2-knockout cells (U2OS ΔΔ); its replication is also less efficient when cells are treated with siRNAs targeting G3BPs (208). Interestingly, in NIH 3T3 cells, deletion of either the RRM or RGG motif in G3BPs abolishes the ability of these motifs to support CHIKV replication. However, in U2OS (human osteosarcoma) cells, the deletion of the RRM is tolerated (174). Similarly, if repeated motifs in nsP3 of CHIKV that interact with G3BPs are deleted or mutated, the virus is not viable unless it can recover at least one of the interaction motifs either by reversion or by recombination events, i.e., by regaining the ability to interact with G3BPs (174,181). Alternatively, as highlighted above, the dependence of CHIKV on G3BPs can be reduced by mutations that slow down the processing of the 1/2 site in the ns-polyprotein. The mechanism of action of such changes is not fully understood. Clearly, these changes do not restore binding to G3BPs. It is likely that they prolong the half-life of the P123+nsP4 complex, an early replicase responsible for negative-strand RNA synthesis. This may alleviate the defect – which is clearly at the stage of negative-strand RNA synthesis – caused by the lack of nsP3/G3BP interactions.

There are three types of G3BP/nsPs complexes at the plasma membrane at the early stage of viral infection. The first one contains only G3BPs and unprocessed P123 polyprotein. This complex is presumed to interact readily with viral G RNAs. The second type of complex is composed of P123, G3BPs and viral ssRNAs; some small amounts of dsRNAs can also be detected. Only the third type of complex contains full-sized dsRNAs, indicating ongoing viral RNA replication (85) (Figure 11). It has also been shown that G3BPs can interact with the 40S ribosomal subunit in the nsP3/G3BP complex, possibly explaining why nsPs and dsRNAs are not colocalized and indicating that G3BPs may play a role in bridging viral RNA replication and nsPs translation (207,208). However, it is difficult to combine all this information into a model that explains all the existing findings. One of these models is shown in Figure 11. Although this model is elegant and does explain the crucial role of G3BPs in alphavirus replication, it does not provide a clear answer as to why different Old World alphaviruses have drastically different sensitivities to G3BPs depletion.

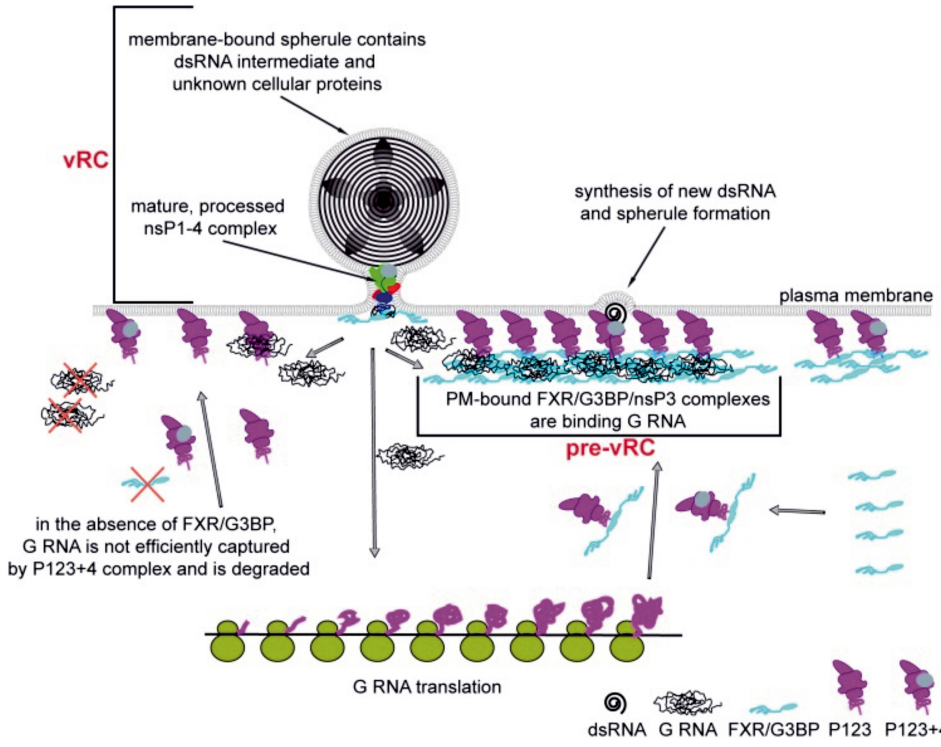

Figure 11. Model of G3BP-and FXR-mediated alphavirus replication complex formation. In the early stage of alphavirus infection, the translated or processed nsP123+nsP4 complex interacts with G3BPs or FXR and recruits G RNA, forming a previral replication complex at the plasma membrane, in which the first molecule of dsRNA is synthesized and used as a template to generate new G RNAs. As infection progresses, additional G RNA is produced and recruited for the translation of the next round of P123+P4 or recruited to pre-vRC for replication. Figure is adapted from (174).

Thus, the exact role, or more likely multiple roles, of G3BPs in alphavirus replication remain to be revealed. The same is true – and to an even greater extent – for other host factors involved in alphavirus replication. Studying the effects of these host factors is challenging because, unlike G3BPs, the depletion/knockout of any of these factors has only a small (often poorly detectable) effect on alphavirus replication. Furthermore, host proteins may function differently in the case of alphaviruses, in different cell types, at different stages of infection and so on. Thus, there is little hope to obtain a complete picture of host factors and their multiple roles in alphavirus replication in general. However, as the data accumulate, our understanding of the roles of these factors in specific viruses under specific conditions will undoubtedly continue to improve.

1.4. Assembly of alphavirus replication complexes

As described in Section 1.2.1, alphavirus RNA replication is associated with spherules (Figure 12A), which are membrane invaginations formed by all alphaviruses analyzed thus far. One study showed that spherules were equally produced in cells infected with wild-type SINV or its mutants with uncleavable 2/3 or uncleavable 1/2 and 2/3 sites. These findings suggested that P123 (or nsP1+P23), nsP4, and the template determine spherule formation (85). Indeed, no spherule formation has been observed when nsPs are expressed as individual molecules; thus, to the best of our knowledge, the requirement for uncleaved P123 (or, at least, P23) seems to be absolute. However, the requirement for templates and the ability of nsP4 to perform RNA synthesis are less clear. These steps are clearly needed for the formation of functional spherules. However, spherule-like structures also formed when nsP1 and nsP4 were expressed together with uncleavable P23 in the absence of a template (131).

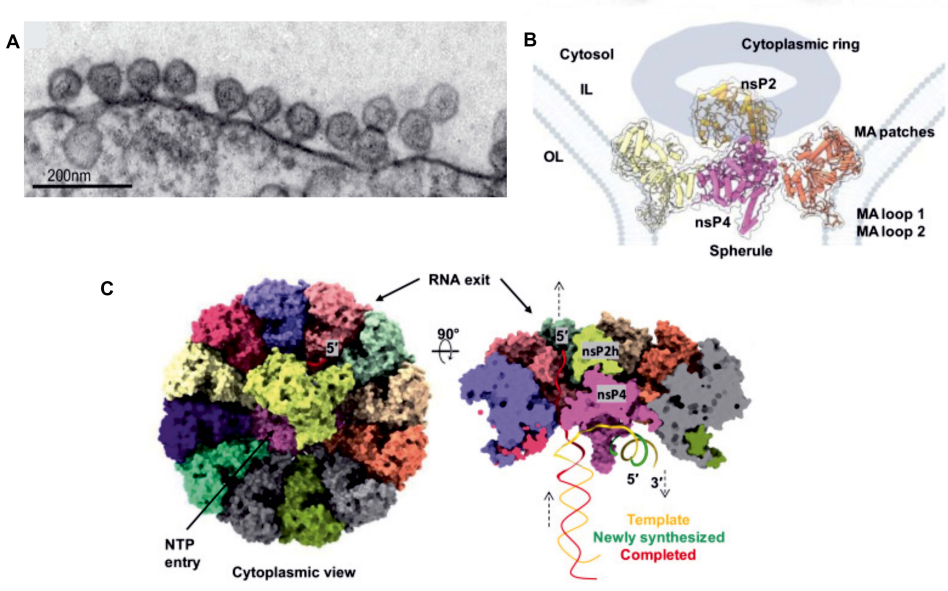

Figure 12. Spherules in BHK21 cells infected with CHIKV (A), model of the membrane-bound replicase complex based on tomography analysis (B), and model of the core of the viral replicase complex performing RNA replication (C). The figure is adapted from (90,211).

At early times post infection, the spherules and dsRNAs of alphaviruses associate with the plasma membrane (85,86). It is also clear that all four nsPs are components of spherules (or, at least, associated with them); however, the stoichiometry of the nsPs has not been determined. Considering that negative-strand RNAs are synthesized when P123 is not cleaved, it is reasonable to speculate that, in a forming spherule, nsP2 and nsP3 can be present in the same copies, similar

to nsP1. For the SINV mutant unable to process P123, this clearly must be the case. However, for a wild-type virus, the situation may be different: at least two molecules of P123 must be present (single P123 cannot be processed into mature proteins as cleavages of 2/3 site occur *in trans*), but what of the remainder? As a significant part of nsP2 localizes to the nucleus and nsP3 interacts with G3BPs to form nsP3 condensates, it is highly likely that the amounts of nsP2 and nsP3 in the forming spherules are less than the amount of nsP1. Considering the instability of nsP4, it is expected to be present in the lowest amount; most likely, a single copy is present in the pore formed by the dodecameric ring of nsP1. Regarding nsP2 and nsP3, it is not obvious where additional copy (or copies) of nsP2 localizes, and regarding nsP3, we do not know anything about its localization other than that it must localize to the cytoplasmic side of the nsP1 ring; this can be deduced from its function: it must be available to interact with known host factors or other unidentified components.

As infection proceeds, nsP1 is further cleaved from P123, immediately followed by the final cleavage between nsP2 and nsP3. Due to the rapid processing, it is highly likely that only one type of mature replicase complex is formed during this period. However, the arrangement of viral nsPs in this mature replicase complex was unknown until a recent study revealed the structure of the mature RNA replicase core (90). This structure is formed both by purified recombinant proteins and inside CHIKV-infected cells; in both contexts, nsP1, nsP2, and nsP4 form a disk-shaped base with a central protrusion. The stoichiometric ratio of the proteins is 12:1:1 (Figure 6); and a single copy of nsP2 is docked above a copy of nsP4, which is slotted within the central pore formed by the nsP1 dodecameric ring. The oligomeric α-helical bundles (residues 335–364) of nsP1 interact with and stabilize nsP4 at the center of the dodecameric ring. Ten copies of nsP1 (out of 12 copies) recruit flexible loops (residues 365–380) to tightly hook onto nsP4; accordingly, the structure contains two channels that probably serve as importers of G RNAs for negative-strand RNA synthesis and as exporters of newly made G and SG RNAs. The NTD of nsP2h slips at the interface between nsP4 and the flexible loop of nsP1, where nsP1, nsP2, and nsP4 interact in three ways. Considering these interactions, nsP2h may function by pulling out the newly made G RNA from the spherule (Figure 12C); however, whether this is indeed the case remains to be confirmed.

Thus, we obtained an excellent overview of the matured spherule neck structure. It is also clear what is inside of spherule –a single copy of viral dsRNA. However, the functional replication conformation complex should contain nsP2p (probably located at the top of nsP2h), nsP3 and host factors (including G3BPs). However, those components are not present at all in the replication core. Intriguingly, there is a cytoplasmic ring that is loosely connected to the nsP1 ring. It is logical to assume that this structure is made of nsP3, host factors, and different RNAs. However, the identity of the exact components of the cytoplasmic ring and their location in this structure have yet to be confirmed (Figure 12B). In addition to these structures, contiguous linear density was observed between the crown of the replicase core and the center of the cytoplasmic ring; these structures may be

transported RNAs or even host factors needed for viral RNA synthesis. A clear structure of this density was not obtained due to the shelter formed by the 12 copies nsP1. A recent cryo-EM study showed that dsRNA is evenly distributed in CHIKV spherules, with several conformations in a preferred orientation, aligning orthogonally to points from the spherules' neck into its lumen (212). Additionally, and rather surprisingly, nsPs not included in the spherules were found to form membrane-associated complexes that have the same conformation as the replication complexes (12 copies of nsP1, one copy of nsP2, one copy of nsP4). In all respects, the replicase core appears to be fully functional but is not associated with the cytoplasmic ring or connected to the spherule. These complexes are likely formed at the later stage of CHIKV infection, when an excessive amount of nsPs is likely produced via a nonreplicative (P1234 to P12 and P34) processing pathway. Clearly, the old assumptions that products formed using this pathway do not properly interact with each other and function as individual proteins are not entirely true. It is unclear which fraction of nsPs is truly "free" and how much is presented in these complexes. It is also probably incorrect to label these as "nonfunctional". They do not synthesize viral G or SG RNAs but are likely (similar to their counterpart assembled in test tubes) to perform some RNA synthesis. Even if not they may still do something different from truly "free" nsPs, whether the difference involves an enzymatic function, actions to counteract some host antiviral mechanisms, or something else entirely.

Overall, in the last year and half, our understanding of alphavirus replicase complexes has greatly improved. It is clear that replication complexes are dynamic and complex. It remains unknown whether the same replication complex undergoes different conformational changes (which is probably the case) or whether new types of replicase complexes with different arrangements of viral components are generated in different states of infection and used to accomplish viral RNA replication. Furthermore, the exact conformations of different forms of replicase complexes, most importantly, those of early replicase complex, remains elusive.

1.5. *Trans*-replication system of alphaviruses

The nsPs (and their precursors) of alphaviruses can not only form replication complexes using viral genomic RNAs but also efficiently interact with defective interfering RNAs (DI RNAs) that are produced during viral infection and harbor deletions in protein-encoding regions. The ability to use template RNA *in trans* has been used to design helper RNAs for the alphavirus replicon, as well as *trans*-replication systems (213,214).

The *trans*-replication system was first established for SFV in BSR 7/5 cells (a derivative of BHK cells stably expressing T7 RNA polymerase). In this system, the ns-polyprotein is expressed from a noncapped transcript made by T7 RNA polymerase and translated using the internal ribosomal entry site of the encephalo-

myocarditis virus (IRES of EMCV). The noncapped template RNA is also produced by T7 RNA polymerase and is designed to contain all the *cis*-elements that are crucial for viral RNA replication. Thus, unlike the mRNA of replicase (which lacks these elements), the template RNA can be recognized, transcribed and replicated by replicases. In other words, the SFV template RNA is a mini-genome that contains all the CSEs necessary for replication. As most protein-encoding regions of the alphavirus genome do not contain the sequences required for RNA replication, they can be replaced by sequences encoding marker proteins. This approach greatly simplifies the monitoring and quantification of replicase activity instead of measuring RNA copy numbers, one can simply quantify the expression levels of marker proteins. Two marker genes were used in the original SFV *trans*-replication system: *Renilla* luciferase (Rluc) was used to replace most of the ns-protein coding region, while ORF2, which normally encodes structural proteins, was replaced with tomato fluorescent protein. This system also included a negative control—a plasmid expressing the SFV replicase that was made inactive by the introduction of mutations into the polymerase motif of nsP4 (GDD to GAA). It was found that the system is highly active; in the presence of active (wild-type) replicase, a large increase in marker protein expression was obtained. Partly because of the low background, noncapped template RNAs generated by T7 RNA polymerase are translated much less efficiently than SFV replicase-generated positive strand RNAs that have 5' cap structures. Furthermore, the replication of template RNA, as well as the formation of replicase complexes, was visually confirmed by the presence of fluorescent tomato protein expressed from replicase-generated mRNA corresponding to the SG RNA of the virus (i.e., made using the SG promoter). These properties indicate that the *trans*-replication system of alphavirus is an efficient tool that can be used for various studies. For example, it can be used to quantify the replication levels by replicases harboring different mutations or to analyze the impact of sequences included in template RNA for viral RNA synthesis. Compared to the use of a modified virus genome, this assay is much easier. Uncoupling of replicase protein expression from RNA replication allows us to study mutations that, when included in the genome of a virus, cause a lethal phenotype. Finally, the system is stable; i.e., unfavorable mutations cannot be reversed, pseudoreverted or compensated for by second-site mutations. In short, it is a rather "clean" system.

The T7 RNA polymerase-based SFV *trans*-replication system was used in several important studies. For example, it was used to study *cis*-elements critical for viral RNA replication. Both the 5' UTR and the 3' UTR were found to be needed for the synthesis of negative-strand RNAs (215). These data are consistent with earlier findings from a replicon-vector-based study (216) confirming that the *trans*-replication system is highly reliable. However, there is also a significant limitation: the system requires the expression of T7 RNA polymerase, which means that the process is restricted to BSR cells only or that cells must be cotransfected with an expression plasmid of T7 RNA polymerase, a replicase expression plasmid and a template RNA encoding plasmid. This requirement could be a

challenge for certain cells that are difficult to transfect. To overcome this limitation, a second version of the *trans*-replication system was generated, in which a CMV promoter (human cytomegalovirus immediate early promoter) was used instead of the T7 promoter to produce template RNAs and mRNAs for the replicases of SFV and CHIKV. In this version, both replicase mRNA and template RNA are generated in transfected cells using cellular polymerase II (217,218). This system is both efficient and easy to use; however, it was found to have one problem: a high background of the first marker (the marker used to replace the nonstructural region). The reason for this is simple—the transcripts generated in the nucleus by RNA polymerase II are capped and efficiently translated. This makes monitoring replication by quantifying the increase in the expression of the first marker difficult or impossible, and only the second marker—expressed almost exclusively from replicase-generated "subgenomic" RNA—remains informative. Not surprisingly, this is a common feature of all the constructs in which RNA polymerase II is used to express the template RNA; a high background is observed in both mammalian and mosquito cells. To address this concern, an updated *trans*-replication system was developed using cell-specific (*Homo sapiens, Aedes albopictus,* and *Aedes aegypti*) RNA polymerase I-transcribed template RNAs (122). Here, the sequence corresponding to the template RNA is placed downstream of a cell type-specific RNA polymerase I promoter. The template RNA constructed using this promoter consists of a 5' UTR followed by a region encoding the first 77 amino acids of nsP1 (for SFV and CHIKV; for some other alphaviruses, longer regions were used) fused to a firefly luciferase (Fluc) reporter, followed by an SG promoter controlling the expression of the *Gaussia* luciferase (Gluc) marker and a truncated 3' UTR followed by a poly (A) tail (Figure 13).

As this *trans*-replicase has become a "workhorse" that was used in a number of studies (over 20 published and many in preparation), we have adopted specific terms to describe its activities. The synthesis of full-length (i.e., entire template) RNA was termed "replication" because, in this model, this RNA serves as a proxy for the viral genome. The "replication" efficiency was evaluated by the activation (boost) of the first marker (Fluc in Figure 13), i.e., the ratio of its activity in the presence of active replicase (or replicase variant under investigation) to its activity in the presence of polymerase-inactive replicase (harboring GDD to GAA mutation). Following the same logic, the synthesis of mRNA using the SG promoter included in the template RNA was termed "transcription", and the activity of this process was measured by boosting Gluc (second marker in Figure 13) activity. Collectively, all RNAs generated by *trans*-replicase were termed "viral RNAs", strictly speaking, they are not identical to viral G and SG RNAs, nor are they generated by viruses. For simplicity, these terms are used in the subsequent sections of the thesis.

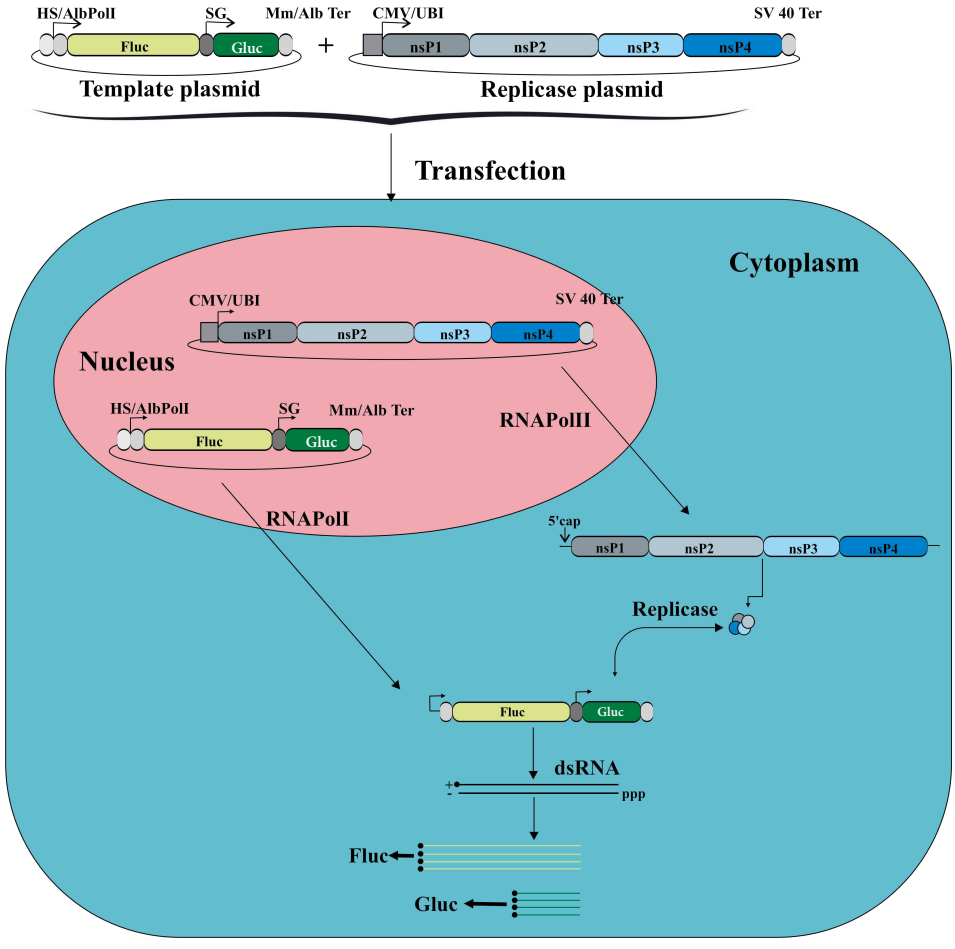

Figure 13. Overview of the alphavirus *trans*-replication assay. Cells are cotransfected with a plasmid encoding alphavirus replicase proteins and a plasmid used for the transcription of replication-competent RNA. In the nucleus, the replicase plasmid is transcribed using RNA polymerase II; however, for the template plasmid, RNA polymerase I is used. These transcripts are transported into the cytoplasm where replicase mRNA is translated into replicase proteins. These proteins can interact with transcribed template RNA and synthesize negative-strand RNA; this process is followed by the synthesis of capped G RNAs and SG RNAs, from which Fluc and Gluc are expressed. The CMV promoter (human cytomegalovirus immediate early promoter) is used for a replicase plasmid in mammalian cells; the Ubi promoter (*Aedes aegypti* polyubiquitin promoter) is used for insect cells. For template plasmids, truncated promoters from HSpolI (*Homo sapiens* RNA polymerase I) and AlbpolI (*Aedes albopictus* RNA polymerase I) are used.

For all positive-strand viruses, including alphaviruses, the viral genome cannot simultaneously serve as the mRNA for nsPs production and as a template for the synthesis of negative-strand RNA. This process will inevitably result in the collision of replicases with ribosomes that are translating the genome. Furthermore, in real virus infection, any defect in viral RNA replication will reduce the copy number of viral RNAs, which in turn decreases the amount of translated nsPs, and this reduction in the amount of nsPs will further reduce viral RNA replication, and so on. In other words, for a real virus, the expression of nsPs is tightly coupled with viral RNA replication. As mentioned above, this situation creates a challenge when studying the impact of introducing mutations into replicase proteins. In real viruses, small changes in replicase activity lead to small changes in the amount of RNA, which results in other small changes. The ultimate outcome may be a drastic effect. This makes comparing the effects of different mutations very difficult; furthermore, viruses respond to drastic effects by reversions of the original mutations (or pseudoreversions or second-site mutations—whatever is easiest and most efficient). In many cases, this approach is informative (especially for the mapping different second-site mutations) but makes analyzing the impact of the original mutation difficult or impossible. In the *trans*-replication system, however, the expression of nsPs is uncoupled from template RNA replication. Hence, the translation of nsPs does not depend on whether the template replicates well. With a real virus, there is always a possibility that a mutation affecting the amino acid sequence of the replicase protein also affects some RNA elements –, for example, the SG promoter (90,219,220) or some high-order RNA structures of unknown function. In the *trans*-replication system, the RNA structures (included in the template construct) and viral proteins (expressed by the replicase construct) can be manipulated separately. For these reasons, a *trans*-replication system has provided a convenient and reliable method for quantifying viral G and SG RNA production and characterizing elements (both in replicase and templates) affecting their synthesis. However, models remain good and valuable only as long as researchers remember that they are models. For this reason, the standard practice in our laboratory is to verify the most crucial findings found by a *trans*-replication system using an infectious virus (unless such a virus is considered potentially dangerous) and often in other ways. Combining the results of two approaches allows us to minimize the shortcomings of virus-based experiments and of our model and to obtain reliable data.

The important benefit of the *trans*-replication system is that it can be used to study dangerous pathogens (such as CHIKV and EEEV) using normal cell culture conditions without the need for a high biosafety laboratory. Furthermore, as shown in our laboratory, in many cases, a *trans*-replication assay can be used instead of constructing chimeric viruses that may, at least in theory, have unpredicted dangerous properties. The system has very high sensitivity and high reproducibility, making it a method of choice for different studies. Thus, the system has been used to study the necessary components for replication complex formation (85), the impact of mutations introduced into nsPs on viral RNA synthesis in both mammalian (221) and mosquito cells (222), the identification of *cis*-acting elements

that are crucial for replication, the feasibility of tagging nsPs and the effects of this manipulation on viral replication (218), as well as the impact of the host factor G3BPs on alphavirus RNA replication (181). A large panel of studies performed in our laboratory has been dedicated to analyzing the compatibility of different alphavirus nsPs (219,220) and the cross-utilization of alphavirus template RNA (202). Although this system has been extensively used, it has, as highlighted above, several limitations. For example, its sensitivity is limited by the properties of the transcribed template RNA (i.e., RNA that is used to start the replication process) and its interaction with nsPs. There are also limitations caused by host cells. For example, in insect cells (such as *Aedes albopictus* C6/36 cells), the replication level remains much lower than that in mammalian cells. Even the use of the noncapped template RNA produced by *Aedes albopictus* RNA polymerase I does not result in large increases in the amounts of replication markers (122). Furthermore, uncoupling nsPs translation from template RNA production can lead to false replication signals in this system; i.e., such mutations can be classified as attenuating, while in the context of the virus genome, the same mutations can render the virus nonviable (132).

2. AIMS OF THE STUDY

The alphavirus nsP2 has been extensively studied. However, because of its multiple functions, the understanding of its role in virus replication is incomplete. The protein has RNA helicase, NTPase, RTPase, and protease activities; it is also involved in the binding and packaging of viral RNA into virions and is responsible for inhibiting host cell transcription and counteracting the innate antiviral immune response. The different functions of nsP2 are well coordinated and linked with each other, complicating further studies. While the structure of nsP2 can provide insights into these issues, only the structure of the nsP2 protease region has been determined. Its role in interactions with other replicase proteins is poorly understood. The functions of other replicase subunits, such as nsP3, are even more enigmatic. It is known that nsP3 interacts with multiple cellular factors, including G3BPs; however, it is not known whether these host factors need to interact with nsP2 or whether they can perform their pro-viral activities even if they are bound to other replicase subunits. We chose nsP2 as a target since a previous study indicated that G3BPs can colocalize with nsP2 (208).

The studies included in this thesis were focused on the following aims:

1. To examine whether CHIKV replication could be influenced by mutations designed to disrupt the stacking interactions between nsP2h and RNA14 and affect the NTP binding site. To this end, I participated in a study resulting in the resolution of the crystal structure of the CHIKV nsP2 helicase domain. My aim was to identify the amino acids that are crucial for viral replication using a structure-guided mutagenesis method.
2. To analyze the effects of the flexibility of the nsP2 interdomain linker on CHIKV replication and the underlying mechanism(s). Again, I participated in a study that resulted in the successful determination of the 3D structure of full-length nsP2. My aim was to investigate the importance of the unstructured flexible linker connecting the nsP2h and nsP2p regions.
3. To investigate whether the activity of CHIKV replicase mutants could be increased in human cells and in insect cells by inserting G3BP/Rin binding motifs into nsP2 flexible regions and, if so, whether this increase in activity was due to the presence of G3BPs/Rin.
4. To determine whether inserting G3BP/Rin binding motifs into flexible regions of nsP2 could restore the replication of CHIKV lacking the native G3BP/Rin binding motifs in nsP3 in mosquito and vertebrate cells.

3. MATERIALS AND METHODS

A detailed description of the materials and methods used in this study is available in the corresponding sections of publications I, II, and III. Therefore, only the principles of the main methods and the rationale behind the use of these methods are discussed below.

Trans-replication assay (Publications I, II and III)

The principles of the *trans*-replication assay are described in Section 1.5. (Figure 13). In this study, human (HSPolI-Fluc-Gluc) and *Aedes albopictus* (AlbPolI-Fluc-Gluc) RNA polymerase I promoter-based plasmids were used to express the replication-competent template RNAs of CHIKV in U2OS (human) and C6/36 (*Aedes albopictus*) cells, respectively. This design was selected on the basis of the low background of markers from noncapped template RNAs formed in the nuclei of transfected cells and the high boost in reporter signals in the presence of active replicases. This approach was essential because we intended to analyze the activities of replicases harboring mutations, some of which were predicted to eliminate replicase activity or drastically reduce it, and maximal sensitivity of the systems was necessary to distinguish between these phenotypes. The plasmids expressing the ns polyproteins containing the CMV (CMV-P1234) or Ubi (Ubi-P1234) promoter were used in U2OS and C6/36 cells, respectively. In U2OS-derived double-null ΔΔ G3BP1/2-knockout cells (U2OS ΔΔ), to determine the impact of the presence or absence of G3BPs on the activities of mutant replicases, we used a plasmid (CMV-G3BP1) to express the missing G3BP protein. In these experiments, the cells were cotransfected with replicase-expressing plasmids (CMV-P1234 or its mutant variants), template plasmids (HSPolI-Fluc-Gluc) and the G3BP1 expression plasmid.

The basic setup of the *trans*-replication assay used in this study is illustrated in Figure 14. Here, all *trans*-replication assays were carried out in 12-well plates with cells at approximately 80% confluence (at the moment of transfection). Again, selections were made to achieve the maximal sensitivity of the system: as was evident from other studies performed in our laboratory, the sensitivity of the *trans*-replication assay performed using 24-, 48- or 96-well plates was lower. For many experiments, the other plates are acceptable (as activities are still very high) but for the studies included in this thesis, the setup with the maximal sensitivities was necessary despite the high consumption of materials and reagents. The experiments were performed as follows: two plasmids (one expressing replicase proteins and another encoding template RNA) were cotransfected into cells; in some experiments, the G3BP1 expression plasmid was also included in the transfection mixture. All the experiments included a positive control (a plasmid expressing the wild-type replicase of CHIKV) and a negative control (a plasmid expressing the polymerase-inactive form of the CHIKV replicase). After transfection, the cells were cultured either at 37 °C with 5% CO_2 for 18 hours (for U2OS and

U2OS ΔΔ cells) or at 28 °C for 48 hours (for C6/36 cells). After the incubation period, the cells were lysed in commercial lysis buffer, and Fluc and Gluc signals were measured using a dual-luciferase kit and GloMAX SIS luminometer. The Fluc and Gluc activities in all cells transfected with replicase variants under investigation were normalized to those in the control cells (i.e., cells cotransfected with the nonfunctional replicase expression plasmid, CMV/Ubi-P1234^{GAA}, and corresponding template RNA plasmid). The observed "boosts" of Fluc and Gluc activities were used to estimate the replication and transcription activities of the replicase under investigation. At least three biological replicates were performed for each experiment.

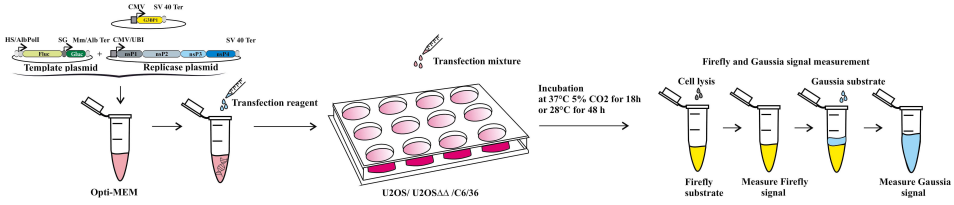

Figure 14. Schema of the *trans*-replication assay used in Publications I, II and III. Replicase and template RNA plasmids were cotransfected into U2OS or C6/36 cells. In U2OS ΔΔ cells, the G3BP1 expression plasmid was also included in some experiments. The Fluc and Gluc signals were measured using a dual-luciferase kit and GloMAX SIS luminometer.

Western blotting (Publications I, II and III)

Western blotting is a routine technique for identifying a target protein in a mixture of proteins (cell lysates, protein mixtures obtained in pull-down experiments, etc.). We used this method to determine the expression levels of CHIKV nsPs and CP in infected cells and to document the pull-down of G3BP/Rin by modified nsP2 proteins. Western blotting also allows reasonably accurate quantification of the amounts of protein(s) of interest in the analyzed samples. A major limitation of this method is the need for high-quality antibodies to detect the protein of interest. Sadly, commercially available antibodies often do not perform as well as expected due to batch to batch variation, damage during transport or storage, etc. Why is this important? Sometimes the variation in antibody quality makes experiments difficult to reproduce. This is not a flaw of the data but, at least in this case, a limitation due to the quality of the available tools.

For the western blot experiments, the cells were transfected and cultured for the appropriate time, washed with PBS, and lysed with Laemmli sample buffer, and proteins were denatured by heating the sample at 100 °C for 5 min. An appropriate amount of lysate (selected to obtain a satisfactory signal and without overloading the gel) was subjected to SDS–PAGE on 10% or 12% gel. The separated proteins were subsequently transferred to PVDF (polyvinylidene difluoride) membranes and detected using primary antibodies against CHIKV CP or nsPs (in

house), G3BP1 or V5-tag. An antibody against β-actin of mice (which also recognizes β-actin of humans, hamsters and, somewhat surprisingly, *Aedes albopictus*) was used as a loading control. Then, the membranes were incubated with appropriate secondary antibodies conjugated to fluorescent labels designed for detection using a LI-COR Odyssey Fc imaging system (Figure 15), which was used to visualize the analyzed proteins.

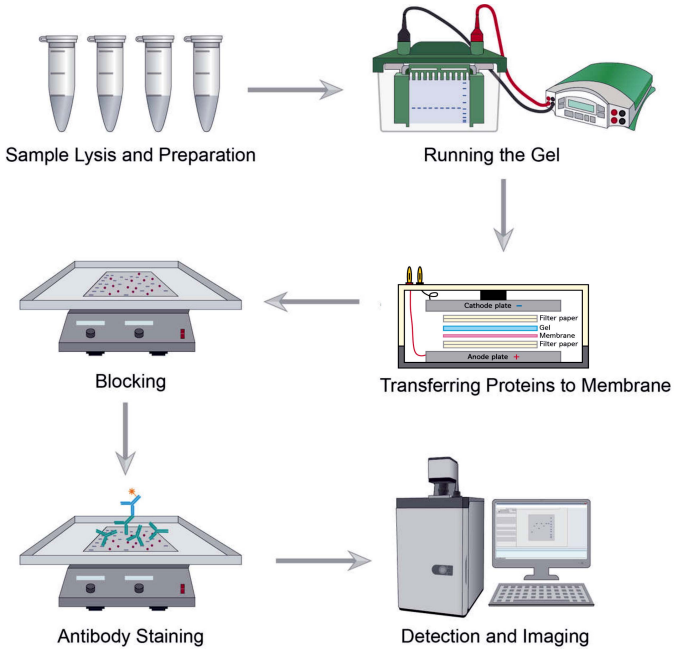

Figure 15. Western blotting workflow. The obtained cell lysates (or pulled-down proteins) were subjected to SDS–PAGE and subsequently transferred to PVDF membranes. Next, primary antibodies were added, followed by washing and the addition of secondary antibodies. Finally, the proteins were visualized with a LI-COR machine. The image was adapted from https://www.creativebiolabs.net/immunoblotting.htm.

Pull-down assay (Publication III)

A pull-down assay is a method used to detect protein–protein interaction. In the pull-down assay, the bait protein (in our experiments, nsP3, nsP2 or its variants) was tagged with EGFP, allowing capture via EGFP-binding magnetic beads. As the pull-down was carried out under nondenaturing conditions, no crosslinking was necessary. The washing of the beads removed the nonspecific binding proteins; therefore, the bait protein and the proteins interacting with it (such as G3BPs or Rin), were pulled down (Figure 16).

The following setup was used for the assay. U2OS cells were transfected with plasmids expressing EGFP-nsP2 or its variants. For C6/36 cells, a plasmid expressing V5-tagged *Aedes albopictus* Rin/Rasputin was included in the transfection mixture because a primary antibody targeting the endogenous Rin protein

was not available. After a selected incubation time, the cells were lysed in non-denaturing lysis buffer, and cell debris was removed by centrifugation. Then, the supernatant was incubated with preequilibrated EGFP-binding magnetic beads, followed by washing with lysis buffer. The bound proteins in the beads were denatured by boiling in Laemmli sample buffer for 5 min and then analyzed via western blotting as described above.

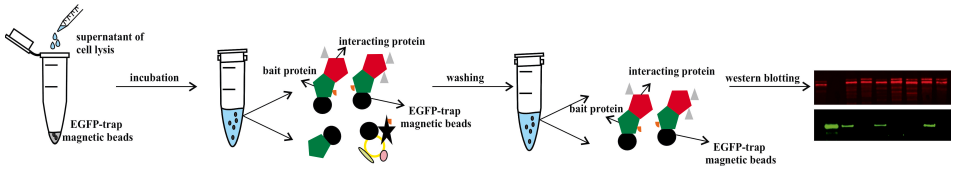

Figure 16. Schema of the pull-down assay. Lysates of transfected cells were clarified by centrifugation, incubated with EGFP-Trap magnetic beads and washed with lysis buffer. Then, western blotting analysis was performed.

Infectious center assay (ICA) (Publications I and II)

The infectious center assay is a method for checking the infectivity of viral nucleic acids: G RNAs, *in vitro* transcripts corresponding to G RNAs, or plasmids containing cDNA from viruses under the control of the eukaryotic RNA polymerase II promoter, most commonly (as in this study) the CMV promoter. Technically, the word "infectivity" may not be strictly correct, because real viral infection involves binding to the host cell, internalization, and particle disassembly. ICA was used to measure the ability of viral RNA to initiate replication and infectious virus formation. Viral RNA was transcribed by cellular RNA polymerase II using plasmids containing wild-type or mutant cDNA from viruses under the control of the CMV promoter. The principle of the assay is simple: the CMV promoter is used by cellular RNA polymerase II, and transcripts that initiate viral RNA replication and ultimately infectious progeny are generated. This method is simple to perform and crucial for evaluating the infectivity of genomes harboring unfavorable mutations. If only the titers of the infectious progeny are measured, much information is lost, and false (or at least flawed) conclusions can be drawn. Frequently, the effect of a mutation is incorrectly understood or missed entirely because viruses in general and alphaviruses in particular tend to generate adaptive mutations that are often capable of restoring virus titers to levels similar to those of wild-type viruses. This happens very quickly, making it difficult to document a small (a few hours) delay in the rescue and spread of mutant viruses without the use of markers and time-lapse recording. On the other hand, ICA can easily identify whether mutations under investigation have no effect on viral infectivity or attenuate it to such a degree that the virus survives by generating adaptive mutations. If the assay suggests the generation of adaptive mutations, the viral stocks can be analyzed for the presence of changes, which are often very informative. For these reasons, we use ICA to determine the true impact of mutations on viral infectivity and to identify viral progeny that potentially contain genomes harboring adaptive mutations.

Briefly, to perform ICA, plasmids containing cDNA from CHIKV (with or without mutations) were electroporated into cells. One tenth of the electroporated cells were subjected to tenfold dilutions and seeded in 6-well tissue culture plates containing nearly 100% confluent BHK-21 cells. After 2 hours of incubation, the cells were overlaid with growth medium supplemented with carboxymethyl cellulose (CMC) and cultured for an appropriate period. The viral RNA infectivity was analyzed by counting the plaques stained with crystal violet. The remaining electroporated cells were plated into a 6-well plate, after which the virus stock was harvested, titrated and further analyzed (if ICA suggested reversions/pseudore versions/adaptations) (Figure 17).

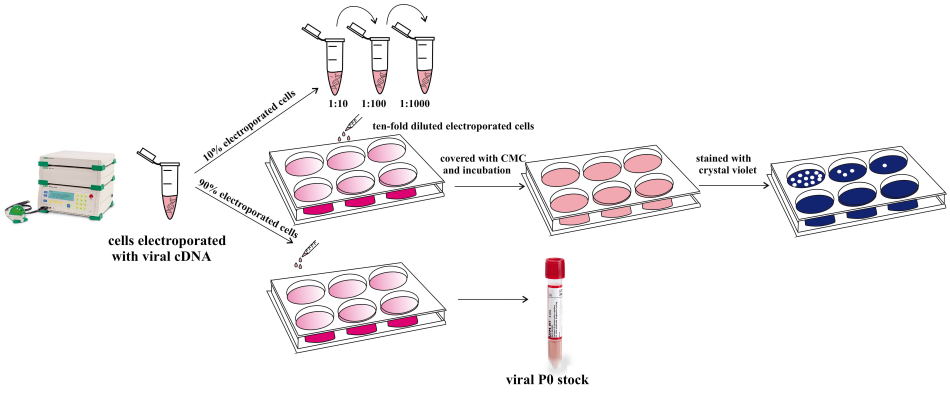

Figure 17. Schema of the ICA. BHK-21 cells were electroporated with plasmids containing viral infectious cDNAs. Ten percent of the electroporated cells were used to prepare tenfold dilutions that were seeded on a 6-well plate with confluent cultures of BHK-21 cells. Afterward, culture medium supplemented with carboxymethyl cellulose (CMC) was added, and the cells were incubated for a specified time (typically 2–3 days), after which they were stained with crystal violet to assess viral RNA infectivity. The remaining 90% of the electroporated cells were seeded on cell culture plates and incubated for 2–3 days (or until cytopathic effect (CPE) development) to obtain passage zero (P0) stocks.

RT–qPCR and RT–PCR (Publications II and III)

Quantitative reverse transcription PCR (RT–qPCR) is a technique that combines reverse transcription and quantitative PCR. This method has a variety of uses, such as quantifying gene expression levels and detecting pathogens such as viruses for the diagnosis of infectious diseases. RT–qPCR is sensitive enough to detect low amounts of viral RNA and is easy to perform. However, RT–qPCR does not determine the number of infectious units but only the copy number of viral RNAs. The infectivity of the virus can be determined by the genome copy number per PFU (plaque-forming unit) ratio: the same number of viral RNA copies can correspond to drastically different numbers of infectious units. This is not an intrinsic limitation of the method; it can also be beneficial. Among other factors, the genome

copy number per PFU ratio may be altered by mutations in the viral genome. RT–qPCR (sometimes in combination with classical virus titration) can be used to analyze the impact of mutations on the infectivity of virions containing mutant genomes. This method is also useful because of its sensitivity, as very low numbers of viral RNA genomes can be accurately detected and quantified. Therefore, we used this method to determine the viral RNA copy numbers of the mutant viruses. RT–PCR is similar to RT–qPCR, except that it yields a normal amplification without quantification. However, we can use this technique to amplify viral genome fragments and use the obtained fragments to analyze viral RNA sequences (Figure 18).

Briefly, for RT–qPCR, viral RNA was purified from harvested virus stocks using a Quick-RNA miniprep kit. The obtained CHIKV genomic RNA was used for the synthesis of cDNA via the use of a First Strand cDNA Kit with random primers. cDNA was subjected to RNA copy number analysis via SYBR Green-based qPCR (using HOT FIREPol EvaGreen qPCR Mix Plus) with a LightCycler 480 II instrument; the primers used were designed to match the nonstructural region, i.e., only viral genomes but not SG RNAs were detected. A standard curve was obtained using different amounts of plasmids containing CHIKV icDNA. For RT–PCR, cDNA was obtained as described above and subjected to normal PCR amplification via DreamTaq DNA polymerase and suitable primers. The obtained PCR products were purified, cloned and inserted into the pJET vector using the cloneJet PCR cloning kit. This step was essential because Sanger sequencing of RT–PCR products is typically not informative; many different sequences are present in the PCR fragment, and viruses can generate adaptive mutations via many mechanisms. The adaptive mutations can be revealed via next-generation sequencing, but we prefer a more classical approach. Subsequently, several purified plasmids (each containing a single variant of the PCR product) were analyzed using Sanger sequencing (Figure 18).

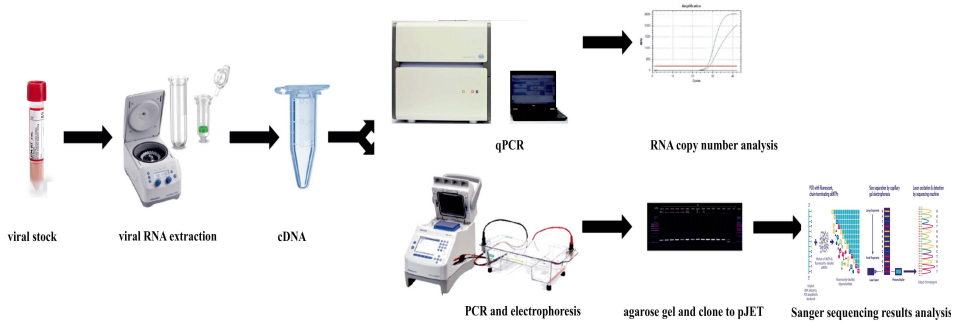

Figure 18. Schema of the RT–qPCR and RT–PCR assays. cDNA was synthesized from the template of viral RNA purified from the obtained viral stocks. To detect RNA copy number, RT–qPCR was used, while standard RT–PCR was used to amplify sequences that were subsequently cloned, inserted into plasmids and analyzed using Sanger sequencing. Figure is adapted from (223).

4. RESULTS AND DISCUSSION

4.1. Mutations that disrupt stacking interactions between nsP2h and RNA or affect NTP binding site have detrimental effects on CHIKV RNA replication and infectivity (Publication I)

Despite the central role of nsP2 in alphavirus infection, information about its structure is limited: only structures of the protease parts (nsP2p) of CHIKV and VEEV nsP2 have been published (224,225). It was also clear from functional studies that nsP2h somehow affects the activity of nsP2p (226) and, conversely, that nsP2p is crucial for the RNA helicase activity of nsP2 (135). As nsP2h clearly plays a central role in many functions assigned to the nsP2 of alphaviruses, attempts were made to use homology modeling to obtain insight into its structure. This method was reasonably informative for predicting the structure of the conserved RNA helicase core; however, determining the structure of the N-terminal region of nsP2h, which is unique to alphaviruses, resulted only in low-confidence models (135).

A breakthrough in the analysis of the real conformation of CHIKV nsP2h (residues 1-465) was achieved via experiments performed by a team led by Luo Dahai (NTU, Singapore). They obtained a high-quality recombinant CHIKV nsP2h protein that bound ssRNA and the nonhydrolyzable transition state nucleotide ADP-AlF_4. This complex was crystallized and was the first alphavirus helicase structure to be resolved at high resolution. nsP2h was found to consist of multiple domains: the N-terminal domain (NTD), the stalk α-helix, the accessory domain 1B of superfamily I (SF1), and two similar Rec-A-like domains (RecA1 and RecA2) (**Publication I, Figure 1B, 1C**). The NTD-stalk-1B domain forms a top cover for the ssRNA-binding groove by interacting with the 3’ region of the bound RNA and RecA1 domain. The 14-mer ssRNA corresponding to the conserved 3’ end of the genomic RNA (RNA 14; sequence 5’-GUUUUUAAUAUUUC-3’) lies at the top of RecA1 and RecA2. RNA14 was used because “it made sense”; however, the protein could bind any other RNA with similar efficiency. ADP-AlF4 tightly bound to the ATPase active site located between RecA1 and RecA2. In addition, a region connected the bottom parts of RecA1 and RecA2 (hence, this region was termed the “connector”) to form the ATP entry tunnel (**Publication I, Figure 1B, 1C**).

One of the most striking features of the resolved structure of nsP2h was the unique mode of protein and RNA interactions. Often, viral proteins bind RNA via the interaction of positively charged amino acid residues and negatively charged phosphates in the RNA. Such interactions could also be observed for the nsP2h and RNA complex. The nucleotides U2-A8 of RNA14 were observed to bind in the 5’-3’ direction in the central groove of nsP2h by interacting with conserved RNA backbone recognition motifs on the top of helicase core domains, including Ia, Ic, III, IIIa, IV, V, and Va. For example, in motif Ia, the positively charged

K211 residue contacted the phosphates U6 and A7, and T378, located in motif V, interacted with the U5 phosphate (motif V plays a role in unwinding DNA in the hepatitis C virus NS3 helicase (227)). However, in addition to these "classical" interactions, three unique stacking interactions were found between nsP2h and the bound ssRNA. Specifically, the aromatic side chain of F287 in motif III intercalates between the U4 and U5 residues of RNA and separates the 5' end of RNA (U2-U4) and the 3' end of RNA (U5-A8); these regions of RNA interact with the RecA2 and RecA1 domains, respectively. Furthermore, the side chains of Y161 and F164 in domain 1B formed stacking interactions with the U6 and A7 residues of RNA, respectively. As seen in the resolved structure, these interactions bent the bound RNA, forming an A-form conformation (**Publication I, Figure 1B, 1C, 1D and 1E**).

In our study, the transition state of ATP hydrolysis was mimicked by ADP-AlF_4, which was observed to be tightly bound to the NTPase active site via interactions between ADP and residues K192 and S193 within the motif I, residue R312 from motif IIIa, and the Mg^{2+} ion, which is essential for RNA helicase activity. Overall, the active site formed a conformation with a water molecule and Mg^{2+} above, AlF_4 in the middle, and an ADP molecule below (**Publication I, Figure 1F**). AlF_4 was stabilized by extensive polar interactions with K192 (motif I), Q283 (motif III), R312 (motif IIIa), and R416 (motif VI) and by interactions mediated by a water molecule (E253, motif II). Thus, many residues are involved in ADP binding; Q283 of motif III is among the most conserved residues in alphaviruses and is likely to be important for ATP hydrolysis, RNA binding, and RNA helicase activities. In addition, ADP binding was observed to involve stacking interactions with residue W450, which lies in the connector (**Publication I, Figure 1F**).

Finding something new and exciting always results in a question—are our findings relevant? After all, the structure of the crystallized protein may not correspond to the dynamic structure of the native protein in a living cell. Therefore, there was a small chance that these interactions were artifacts of crystallization. In addition, it is difficult or impossible to predict what (if any) role these novel types of interactions have in the activity of nsP2. Our colleagues in Singapore performed multiple assays using purified recombinant protein to determine the effect of the recombinant protein on ATPase activity; however, these effects were rather diverse, from the activation of enzymatic activity to its complete elimination (**Publication I, Figure 3**). While it is logical to connect a lack of ATPase activity with lethal phenotype, it is difficult, if it is possible at all, to predict the impact of other changes. Therefore, to determine the functional relevance of the interactions between nsP2h and RNA14 and between nsP2h and ADP-AlF_4, a structure-guided mutagenesis study focusing on stacking interactions and NTP-binding site was performed.

Three mutants (Y161A, F164A, and F287A) were generated to study the effects of stacking interactions between RNA14 and nsP2h on the viability of CHIKV as well as on the activity of its RNA replicase. The importance of the stacking interaction between ADP and W450 from the connector was also studied.

Additionally, mutations were introduced to previously studied residues of the NTP binding site (K192A, Q283A).

First, to examine the effects of these mutations on CHIKV replication, a CHIKV *trans*-replication system was used (**Publication I, Figure 4A**). As expected, replicases containing K192A or Q283A mutations lost the ability to replicate template RNA; both replication and transcription signals were at the background level, and these data are consistent with the role of these residues as critical determinants of NTP hydrolysis and viral replication (228). The stacking interaction of W450 with ADP was also found to be important for CHIKV replication, as the corresponding replicase mutant exhibited a near-background level of replication activity as well as strongly and significantly reduced transcription activity (compared to wt replication). Interestingly, the stacking interaction with RNA14 mediated by Y161 and F164 was critical for CHIKV replication: no replicase activity (similar to what we have observed for K192A or Q283A mutants) was observed if either of these residues (or both) were replaced by alanine residues. In contrast, the F287A substitution had no effect on CHIKV replication; however, a significant decrease in transcription was observed (**Publication I, Figure 4B, 4C**).

As described in the Section 1.5, the *trans*-replication assay is a highly useful and highly relevant model but still has certain limitations. Thus, it was essential to analyze the effects of these mutations on viral RNA replication. For this purpose, a virus rescue (including an infectious center assay [ICA]) was performed. Consistent with the *trans*-replication data, plasmids harboring CHIKV icDNA containing K192A, Q283A, W450A, Y161A, or F164A substitutions had infectivity below the detection limit (less than 2 pfu/μg DNA). The same was true for the F287A mutant, which was surprising since this mutation has very different effects on the *trans*-replication of CHIKV. The difference in the nature of the defect caused by the F287A substitution became more evident when the infectious virus progeny was analyzed. In contrast to other mutants, infectious progeny was obtained for F287A, although the observed titers were much lower than those obtained for the wt construct (**Publication I, Figure 4F, 4G**). Taken together, these findings suggest that the F287A mutation was not lethal but was strongly attenuated. The most likely explanation for the production of infectious progeny was that the debilitating effects of the F287A substitution were compensated for by additional changes in the genome of the mutant virus. This assumption was directly confirmed by sequencing the entire genomes of the rescued viruses. The majority of the analyzed genomes had maintained the F287A mutation and acquired a potential second-site adaptive mutation (in the majority of cases, V410I; less frequently, T358S). In some genomes, a pseudoreversion (F287V) resulting in the replacement of the introduced A287 residue with a more hydrophobic Val residue was observed. Only 10% of the analyzed genomes contained the originally introduced F287A mutation with no additional mutations (**Publication I, Figure 4F**). To further confirm the functional significance of the revealed mutation, the effects of the mutations on CHIKV replication were evaluated again using the abovementioned methods. We found that these mutations had no negative effect on the replication activity of the CHIKV RNA replicase; in fact, in some

cases, this change was a slight (up to 1.5-fold) increase. This finding was not surprising since the replication activity was not compromised by the original F287A mutation. In contrast, all three mutations increased transcription activity, which was similar to that of the wt replicase; i.e., the main defect caused by the F287A substitution was reversed **(Publication I, Figure 4B, 4C**). Consistently, as revealed by the ICA, all three mutant icDNAs had high infectivity (**Publication I, Figure 4F**). Thus, the detected pseudoreversion and second-site compensatory changes were functionally important.

To conclude, we found that ADP-AlF_4 and RNA14 bind to nsP2h using a unique set of interactions that are crucial for CHIKV replication. The mutations K192A and Q283A, which disrupt the NTP binding site, abolish ATPase activity, predictably leading to nonviable CHIKV mutants. Similarly, the W450A substitution, which prevents the formation of stacking interactions with ADP, blocks the ATPase activity of the enzyme and results in a lethal phenotype. Collectively, these data confirm that the NTPase activity of nsP2 in CHIKV is necessary for viral replication. The Y161 and F164 residues in domain 1B were found to form stacking interactions with bound RNA 14. As the mutations at these positions (Y161A, F164A) are located outside conserved NTPase motifs, they do not affect the ATPase activity of nsP2; nonetheless, they completely abolish CHIKV RNA replication (**Publication I, Figure 3A, 4B, 4C**). These findings suggest that these two residues, while dispensable for NTPase activity, may be needed for RNA helicase activity, which depends on the ability of the enzyme to interact with viral RNA. Unlike the lethal effects caused by the Y161A or F164A substitutions, the F287A substitution only reduces but does not abolish CHIKV infectivity; the negative impact is probably due to reduced SG RNA synthesis (**Publication I, Figure 4C**). These findings suggest that the F287 residue might play a role in stabilizing interactions with the region of RNA needed for SG RNA synthesis. The pseudoreversion or compensatory mutations found in viruses harboring the F287A substitution are all located within the conserved helicase SF1 motifs: A287V is located within motif III, T358S is located within motif IV, and V410I is located close to the motif VI. All of these motifs form close contacts with RNAs and affect NTP binding (229). Accordingly, it can be speculated that these mutations might play a role in improving the capture of negative-strand RNA used as a template for SG RNA synthesis, therefore reversing the negative effects of F287A. Indeed, nsP2 has been identified as a component that regulates the SG RNA synthesis in SFV (148). However, the 3D structure of the alphavirus replicase core and the composition of the spherules recently revealed indicate that nsP2 has no direct contact with dsRNA, as nsP4 is located between them (Figure 6). Thus, the impact of nsP2 on SG RNA synthesis is either indirect (mediated by nsP4) or, more likely, due to the movement of the RNA template through the replicase complex. Unfortunately, there is currently no clear information on how the template moves through the replicase to initiate positive-strand RNA synthesis. Such information would allow us to place the F287 residue and positions

associated with compensatory changes (residues 410 and 358) in the correct functional context, helping to understand the exact molecular basis of the biological effects caused by substitutions at these positions.

Overall, the structure of the ternary complex nsP2h-RNA14-ADP-AlF_4 revealed numerous novel and functionally important interactions used for RNA recognition and ATP hydrolysis, and biological assays confirmed that these interactions are critical for CHIKV replication.

4.2. A flexible linker connecting the nsP2h and nsP2p regions regulates CHIKV replication (Publication II)

Combined with the previously reported structure of CHIKV nsP2p (133,224), the work described in Publication I completely covers the structures of the globular regions of nsP2. However, during real infection, nsP2h and nsP2p do not exist: there is only nsP2. Furthermore, the properties and functions of full-length nsP2 are not a mechanical sum of those of nsP2h and nsP2p; full-length nsP2 has functions, such as the processing of the 2/3 site and RNA helicase activity, that neither domain alone can perform (135,226). Thus, nsP2h and nsP2p are clearly connected and function in a coordinated manner. As long as the conformation of the full-length nsP2 remains unknown, there is no structural basis for understanding the functions of nsP2. Hence, resolving the structure of the complete protein is highly important. It is also clear that the task is anything but easy. Overall, nsP2h and nsP2p contain numerous functional domains. The region between them is assumed, based on functional analysis, to be a flexible linker. This can be deduced from several lines of evidence. First, to function together, different parts of nsP2 must interact dynamically with each other. Second, it is known that the insertion of a tag—or even GFP—between the nsP2 N-terminal region and C-terminal region has no obvious negative effects on CHIKV replication. This also suggests that these two regions are connected via a flexible linker, which provides freedom for each region to move to a certain degree (218). However, this flexibility hampers the production of crystals representing full nsP2. Hence, another approach is needed.

As described in Publication II, our collaborators confirmed the presence of a flexible linker using small-angle X-ray scattering (SAXS), which resulted in a reasonably high-resolution structure of full-length nsP2 from CHIKV (**Publication II, Figure 1D**). Interestingly, such a linker is also present in nonstructural protein 3 (NS3) of flaviviruses and hepaciviruses. Like the nsP2 proteins of alphaviruses, these proteins are viral proteases/helicases (although the order of the functional regions is reversed). It has been shown that restraining the flexibility of the linker has a negative effect on the replication of dengue virus and hepatitis C virus (230,231). This raises the following question: what is the role of the flexible linker in CHIKV replication? Is it similar to that observed for flavi- and hepacivirus proteins or something else entirely? To investigate this question, six mutations were introduced to the linker region to reduce (delQ465, delSHQ,

delSHQMT) or increase (insGSG, ins6GS, ins10GS) the linker flexibility. The effects of these mutations on CHIKV replicase activity and on virus infectivity were analyzed using methods similar to those used in the study described in Publication I.

First, the CHIKV *trans*-replication system was used to characterize the effect of mutations in the nsP2 linker region on the activity of the CHIKV RNA replicase. We found that one amino acid deletion (delQ465) had no detectable effect on the replication or transcription of the CHIKV replicase. However, when three (delSHQ) or five (delSHQMT) amino acid residues were deleted, significant decreases in the Fluc and Gluc signals were observed. Furthermore, the effect of larger deletions was more prominent (**Publication II, Figure 2C, 2D**). In contrast, the mutations that increased linker flexibility (insGSG, ins6GS, ins10GS) increased the RNA replication level. The effect was especially prominent for ins6GS and ins10GS, both of which produced a particularly strong increase in Fluc activity. Interestingly, no significant effect on CHIKV transcription was observed (**Publication II, Figure 2C, 2D**). To exclude the possibility that the abovementioned effects are cell type specific (similar to the experiments described in Publication I, human U2OS cells were used for this analysis), the same experiments were performed in C6/36 (*Aedes albopictus*) cells. Overall, the effects observed in mosquito cells were similar to those observed in mammalian cells. Again, CHIKV replicases harboring deletions of three or five linker residues (i.e., delSHQ and delSHQMT mutations) exhibited a significant decrease in transcription activity. In contrast, replicases harboring insertions of six or ten residues (including 6GS and 10GS) had increased transcription levels (**Publication II, Figure 2E**). Unfortunately, we could not analyze the effect of these mutations on replication activity, as the boost in Fluc activity in mosquito cells was not prominent enough and did not correlate well with the level of full-length viral RNA synthesis (202).

To analyze the effects of these mutations on CHIKV infectivity, virus rescue and ICA were performed using BHK21 cells. As we expected, all three insertions slightly increased the infectivity of the corresponding CHIKV icDNA plasmids. In contrast to the increase in the PFU/RNA copy number ratio, the final titers of the obtained virus stocks were somewhat lower than those of wt CHIKV (**Publication II, Figure 3A**), suggesting that the mutants may have defects in the production or release of infectious virions. In contrast, for the delQ465 mutant, a mild decrease in virus rescue efficiency was observed. More importantly, the icDNAs of CHIKV harboring delSHQ or delSHQMT mutations were not infectious. In contrast to the F287A mutation described in Publication I, the lack of infectivity was not attributable to the limited sensitivity of ICA, as the lack of virus rescue was confirmed by the absence of infectious progeny and the lack of viral capsid protein expression (**Publication II, Figure 3A, 3B**). Similar results were obtained when we attempted to rescue the delSHQ and delSHQMT mutants in U2OS cells, excluding the possibility that this lack of rescue was specific to BHK21 cells. Interestingly, in transfected U2OS cells, the expression of a protein

with a higher molecular mass, detectable using an anti-nsP2 antibody, was observed for the delSHQ and delSHQMT mutants (**Publication II, Figure 3C**). To identify this protein, we used corresponding replicase mutants to transfect U2OS cells, and the resulting samples were analyzed via western blotting with CHIKV nsP1, nsP2, and nsP3 antibodies. This analysis revealed that the protein was unprocessed P23 (i.e., it was recognized by antibodies against CHIKV nsP2 and nsP3) (**Publication II, Figure 3D**). The same unprocessed P23 was also observed in a cell-free assay (**Publication II, Figure 4E, 4F**).

Taken together, our data confirm that the linker (amino acid residues 463–476 of nsP2) plays an important role in CHIKV replication. The region is indeed flexible; hence, the nsP2h and nsP2p regions are loosely connected (**Publication II, Figure 1D**). The linker can tolerate the deletion of one amino acid or the insertion of up to 10 amino acids with no negative impact on viral RNA replication. However, deletions of three or five amino acid residues (delSHQ and delSHQMT, respectively) are lethal for CHIKV. Interestingly, the same deletions resulted in only a modest reduction in RNA synthesis in the CHIKV *trans*-replication assay. This result leads to the following question: why do these deletions have such strong effects? The detection of unprocessed P23 might provide insight into this phenomenon. The decreased flexibility of the nsP2 linker region could restrict the access of the nsP2 protease to the 2/3 site, thereby affecting its *trans*-cleavage. This seems likely to hamper the conversion of the early replicase complex into a late replicase complex, leading to a nonviable phenotype. However, in reality, it is unlikely to be simple. CHIKV replicases harboring mutations in the P2 residue of the 2/3 site (completely preventing its cleavage) are highly active (222), and the corresponding virus is viable. Thus, processing defects alone cannot explain the phenotypes caused by delSHQ or delSHQMT. It is likely that other—and likely more crucial—functions of nsP2 are compromised as well. RNA helicase activity is a logical assumption, as this function also depends on dynamic interactions between the nsP2h and nsP2p regions. Interestingly, the helicase activity of nsP2h requires the presence of nsP2p, but simply adding nsP2p to nsP2h is not enough: connection via a linker is needed for the protein to display RNA helicase activity. Although our study revealed that the majority of insertion or deletion mutations had no effect on RNA unwinding, delSHQMT did show decreased RNA unwinding activity (**Publication II, Figure 5A**). Does this result explain the biological effect of these mutations? This question is difficult to answer because the actual role of RNA helicase activity in alphavirus replication is unclear. Thus, it remains possible that these mutations act via other mechanism(s). Currently, we do not have an answer to this question; thus, the exact mechanism responsible for the observed defects remains to be studied.

Increased synthesis of G RNAs in U2OS cells and of SG RNAs in C6/36 cells, as well as the increased rescue efficiency in BHK21 cells, was observed for mutants with increased length (and flexibility) of the nsP2 linker. Again, one may assume that these may originate from the fact that the introduced changes probably give the nsP2 protease more freedom to access the 3/4 and/or 1/2 sites that are normally cleaved *in cis*. However, it is not obvious whether this change would facilitate

the assembly of replicase complexes and increase CHIKV replication. Some positive effects may result from increased cleavage of the 3/4 site. However, the opposite is almost certainly true for the 1/2 site—acceleration of its cleavage is detrimental for alphaviruses (145). More likely, flexibility of the linker is required for the coordination of different functions of nsP2 (and/or polyproteins containing nsP2). The coordination of RNA binding between the helicase and protease regions may rely on the high RNA binding efficiency of the nsP2 protease region (more precisely, the MTL domain) (**Publication II, Figure 6A**). The ability to bind dsRNA may allow nsP2p to form the nsP2p-RNA complex, and the flexible linker might arrange the 5' overhang region of RNA toward nsP2h. Therefore, the revealed nsP2h-RNA structure is formed by binding the RNA in the 5'-3' direction to the central groove of nsP2h (133). However, while this hypothesis was reasonable at the time Publication II was written, it does not fit well with more recent data concerning the organization of the alphavirus RNA replicase core. In the mature replicase, no contact was observed between nsP2 (located on the cytoplasmic side of the spherule) and dsRNA (located inside the spherule). Thus, if this interaction is important, it should occur during spherule formation when nsP2 can make contact with dsRNA. Unfortunately, the structure of the early replicase complex has not yet been determined.

Overall, the flexible linker in the nsP2 protein of CHIKV has multiple functions. It is obvious that this region has evolved to allow optimal molecular gymnastics, enabling nsP2 to perform different activities.

4.3. G3BP/Rin-binding motifs inserted into flexible regions of nsP2 can support CHIKV replication (Publication III)

4.3.1. Insertion of G3BP/Rin-binding motifs into nsP2 increases the activity of CHIKV replicase mutants in human cells in a G3BP-dependent manner

Previous studies have shown that the alphavirus nsP2 has three flexible regions that can tolerate enhanced green fluorescent protein (EGFP) insertion without deleterious effects on viral replication. One of the sites is located in the linker region of CHIKV nsP2 (after residue 466). The position after residue 618 of nsP2 (located between the protease and MTL domains) also tolerates EGFP insertion without influencing nsP2 function or subcellular localization (218). In SINV, the insertion of GFP after residue 8 of nsP2 has no deleterious effects on virus replication (205). Based on these studies, we can insert EGFP into these three regions to study the role of nsP2 in CHIKV-infected host cells. Additionally, in CHIKV-infected cells, nsP2 colocalizes with G3BP2 (208). Moreover, in SINV-infected cells, extracted G3BPs coprecipitate with nsP2 (205). This finding confirms the close (albeit probably not direct) contact between nsP2 and these host factors. Furthermore, the sensitivity of G3BPs depletion has been mapped to the arginine residue located at the P4 position of the 1/2 site, as the replacement of this amino

acid with a histidine residue can partially restore CHIKV replication in U2OS-derived double-null ΔΔ G3BP1/2-knockout cells (U2OS ΔΔ) (181). Considering the role of nsP2 as a protease responsible for 1/2 cleavage, as well as its colocalization with G3BPs, it was obvious that this component of the replicase and G3BPs are connected.

As highlighted in Sections 1.3.3 and 1.3.5, G3BPs interact with the duplicated FDGF motifs in the HVD of nsP3. The mutation of these interaction motifs—hereafter named the F3A mutation—blocks the interaction of G3BPs with the CHIKV replicase complex and has a debilitating effect on the activities of the CHIKV replicase in a *trans*-replication assay (181). This approach creates an easy-to-use assay system: we were able to determine whether the activities of the F3A replicase are increased by the insertion of the G3BP-binding peptide (WT peptide) into different positions of nsP2 and compare the effect with that resulting from the insertion of a similar control peptide that is unable to bind G3BPs (F3A peptide). Therefore, these peptides were inserted into flexible regions of nsP2 (after residues 8, 466, and 618) in the context of the CHIKV F3A replicase. In addition, similar insertions were made into the predicted flexible region of nsP1 and the C-terminal region of nsP4, which are also considered flexible regions **(Publication III, Figure 1A**).

The results obtained for constructs harboring insertions in nsP1 or nsP4 are described in Publication III and are not described here in detail. In the CHIKV *trans*-replication assay, the F3A mutation in the HVD of nsP3 significantly decreased CHIKV replication and transcription in U2OS cells, confirming that the interaction with G3BPs is critical for its replication (**Publication III, Figure 2D**). The insertion of F3A peptides into the nsP1 or nsP4 region was poorly tolerated, and no replicase activity was detected in mosquito cells. In mammalian cells, these insertions further attenuated the already low activity of the F3A replicase. Interestingly, the replicase harboring the WT peptide in nsP1 was more active than its counterpart harboring the F3A peptide, while the reverse was true for insertions made into the C-terminal region of nsP4 (**Publication III, Figure 2D**). The 3D structure of the alphavirus replicase provides clear reasons for this behavior. The C-terminus of nsP4 is actually well folded and localizes inside spherules (90), which explains the poor tolerance of this region for the insertion of any peptide. The ability of this region to interact with G3BPs is clearly unnecessary, as this localization also excludes the possibility that G3BPs could display their proviral activities. Furthermore, interactions with G3BPs are harmful to a replicase, as interactions with G3BPs likely reduce the amount of nsP4 available for interactions with the nsP1 ring. When the nsP4:G3BP complex is incorporated into spherules, the presence of G3BPs likely hampers the interaction of nsP4 with RNA. Thus, our negative data are actually quite informative. The C-terminal tail of nsP1 is disordered and therefore not visible in the 3D structure of the replicase core. However, this region is likely involved in mediating interactions between the replicase core and the cytoplasmic ring structure. This interaction region, in turn, allows this region to interact with G3BPs, hence confirming

the positive impact of WT peptide insertion. However, the interaction is likely far from optimal for G3BPs to display fully proviral activities.

Most of the data were obtained for constructs harboring insertions in the nsP2 region. Insertion of the F3A peptide into three flexible regions consistently led to further reductions in Fluc and Gluc signals compared to those of the replicase consisting of $P123^{F3A}4$, which was used as a control (**Publication III, Figure 2D**). In contrast, when the functional WT peptide was inserted into the three flexible regions of the mutated replicase, a significant increase in activity was observed. The most prominent increase in activity was observed for constructs harboring the WT peptide after residue 618 of nsP2 (**Publication III, Figure 2D**). However, the question remains: was this increase indeed due to the interaction of nsP2 with G3BPs? To determine the correlation between the nsP2:G3BP interaction and the increased activity of replicases, we carried out pull-down experiments using expression plasmids in which the N-terminus of nsP2 (or its mutants) was fused to EGFP. We found that G3BP1 was pulled down by all the mutants of nsP2 harboring the WT peptide but not by their counterparts harboring the F3A peptide (**Publication III, Figure 3B**). Thus, there was clearly a direct interaction. Nonetheless, we still do not know whether the increase in replicase mutant activity was due to this interaction. To determine whether this was the case, we carried out the same *trans*-replication assay in U2OS ΔΔ cells. This experiment revealed that in the absence of G3BPs, the activity of the wt CHIKV replicase was strongly reduced, as previously observed (181). The opposite was true for replicase harboring the F3A mutation; i.e., its activity in knockout cells was greater than that of the wt CHIKV replicase. Consistently, in knockout cells, CHIKV replicases harboring the F3A peptide in different locations in nsP2 presented significantly greater Fluc and Gluc levels than did their WT peptide counterparts (**Publication III, Figure 4A**); i.e., the observed phenotype was completely opposite to what we observed in wt U2OS cells (**Publication III, Figure 2D**). These findings indicated that the insertion of the WT peptide *per se* was more harmful than the insertion of the F3A peptide. Thus, it was highly likely that the interaction of G3BPs with the WT peptide inserted into nsP2 resulted in the increased activities of these constructs in wt cells. To confirm this, we next performed the aforementioned experiments in U2OS ΔΔ cells in the presence of an expression plasmid for G3BP1. As expected, the expression of G3BP1 strongly increased the activity of the wt CHIKV replicase. In contrast, only negligible positive effects were observed if the replicase harboring the F3A mutation in the HVD of nsP3 was used (**Publication III, Figure 4B**). Similarly, only minor effects were detected for the activities of replicases harboring the F3A peptide in nsP2. In contrast, the presence of G3BP1 increased the activity of replicases harboring WT peptide insertions. Again, the most prominent positive effects of the presence of G3BP1 were observed for the replicase harboring the WT peptide after residue 618 of nsP2 (**Publication III, Figure 4B**). A minor boost was also observed for the replicase with the F3A peptide inserted after residue 466 of nsP2. The question is why—as discussed in Section 4.2—the linker region could easily tolerate the inserts, and the 10-amino acid linker even increased both the CHIKV replicase

activity and the virus infectivity. It is likely that the F3A peptide inserted after residue 466 also functions as a flexible linker that can increase the activity of replicase mutants. Therefore, an increase in the replication level was also observed in the presence of G3BP1, which cannot bind the inserted peptide. In contrast, the WT peptide seems to be less flexible than the other peptides, and the corresponding constructs, even in the presence of G3BP1, have relatively modest activity. To clarify this situation, we performed the above-described experiment using a construct harboring a flexible linker (10GS) after residue 466 of nsP2 and the F3A mutation in nsP3. A pull-down experiment performed using nsP2 with an inserted 10GS linker showed that the protein did not interact with G3BP1, eliminating the possibility that any positive effect resulting from this insertion was due to binding to G3BPs. In U2OS cells, the CHIKV replicase harboring the 10GS insertion had higher replication and transcription activities than did the replicases harboring the WT or F3A peptide **(Publication III, Figure 5A**). This trend remained the same for U2OS ΔΔ cells without or with the presence of G3BP1 (**Publication III, Figure 5B, 5C**). Overall, in addition to exhibiting somewhat lower activity, the replicase harboring the F3A peptide behaved similarly to the replicase harboring the 10GS peptide. This confirmed that the F3A peptide inserted after residue 466 acted as a flexible linker and activated the mutant CHIKV replicase regardless of the presence of G3BP1. The small magnitude of the boost caused by the presence of the WT peptide at this location is therefore explained by the difference in balance; i.e., the positive effects resulting from the interaction with G3BPs are counteracted by negative effects that restrict nsP2 flexibility

Taken together, our data revealed that the insertion of the G3BP/Rin binding motifs into flexible regions of nsP2 can increase the activity of mutated replicases, and at least for U2OS cells, these increases in activity are due to the interaction of mutated nsP2 proteins with G3BPs.

4.3.2. Insertion of the G3BP/Rin-binding motifs into nsP2 cannot restore the infectivity of noninfectious CHIKV mutants in insect cells

To investigate whether these effects, similar to those observed in U2OS cells, could also be observed in mosquito cells, the above-listed experiments were performed in *Aedes albopictus* (C6/36) cells. As we expected, the wt CHIKV replicase was highly active, while the activity of the replicase with F3A mutations in the HVD of nsP3 was at the background level (**Publication III, Figure 1E**). All the mutant replicases harboring the WT peptide at different locations in nsP2 had significantly greater transcription activity than did their F3A counterparts. The effects of WT peptide insertion depended on the insertion site used. Again, the most prominent effect was observed when the WT peptide was inserted after residue 618. Thus, the data obtained from mosquito cells were similar to those obtained from U2OS cells, indicating that the binding of nsP2 to Rin is beneficial

for replicases lacking interaction motifs in nsP3. The interaction of Rin with nsP2 proteins harboring the WT peptide at different locations was also confirmed using a pull-down experiment (**Publication III, Figure 3B**).

Interestingly, a significant increase in the activity of the mutant replicase was observed when the F3A peptide was inserted after residues 8 and 466 (**Publication III, Figure 1E**). The increase caused by the F3A peptide inserted after residue 466 was highly likely to be attributable to the same reason as that in U2OS cells, i.e., the inserted peptide functioned as a flexible linker. However, the increase in replicase activity was greater than that in human cells, suggesting the occurrence of additional cell type-specific effects. A very prominent increase in replicase activity was observed for the mutant harboring the F3A peptide after residue 8 in nsP2. Previously, delay or blockade of 2/3 site processing was shown to boost CHIKV replicase activity in mosquito cells but not in human cells (222). As the N-terminal domain of nsP2 is crucial for 2/3 site processing (145,226), it was logical to assume that the observed boost could be due to the insertion *per se* having a negative impact on the processing of the 2/3 site. Indeed, western blot analysis revealed the presence of unprocessed P23 polyprotein in cells transfected with this construct (as well as in cells transfected with the variant harboring the WT peptide) (**Publication III, Figure 1C, 1D**). Thus, for these mutants, replicase activity was also prominently increased in a manner independent of the interaction of nsP2 with Rin.

To investigate whether the activating effects of WT peptide insertion could also be observed in the context of the virus genome, we introduced the corresponding mutations into the infectious cDNA clone SP6-CHIKV and its mutant SP6-3^{F3A}CHIKV, which is noninfectious due to mutations in the G3BP/Rin binding motifs in nsP3. The capped transcripts of the aforementioned mutants were used to transfect C6/36 cells, and the replication of the mutant viruses was documented via western blot analysis of the capsid protein expression (as the capsid protein is translated from the SG RNAs, its expression requires replication of the virus genome). Somewhat unexpectedly, the inserted peptides could not restore the infectivity of SP6-3^{F3A}CHIKV (**Publication III, Figure 6A, 6B**). Thus, the data obtained using viral genomes were different from those obtained using a *trans*-replication assay. As we expected, all mutants with native G3BP/Rin binding motifs in nsP3 could be rescued, and infectious progeny could be produced, regardless of whether the WT or F3A peptide was inserted into nsP2. This finding suggested that the inserted peptide *per se* had no detrimental effects on viral replication. Interestingly, however, the CP expression levels and the RNA copy numbers of the viral stocks were consistently lower for the mutants with the WT peptide than for their F3A counterparts (**Publication III, Figure 6A, 6B**). This finding indicated that, compared with the insertion of the F3A peptide, the insertion of the WT peptide into the CHIKV icDNA had a negative impact on virus replication. It can be speculated that because Rin originally binds to the native motifs in nsP3, the additional binding motifs present in another location may have resulted in competition for binding to this host protein, which is unfavorable for CHIKV replication. Alternatively, or in addition, nsP2 with bound

Rin was unable to perform other biological activities, for example, assisting in the formation of infectious virions.

To summarize, we found that the WT peptide in nsP2 increased the activity of the CHIKV replicase lacking the G3BP/Rin binding motifs in the HVD of nsP3; however, this increase was clearly not sufficient to restore the infectivity of the mutant CHIKV genome in C6/36 cells. There are several possible explanations for this discrepancy. As described in Section 1.5, the *trans*-replication system has several limitations. In this system, the expression of the nonstructural polyprotein is uncoupled from template RNA replication. In the case of viruses, these are linked, and there is a switch from translation to viral RNA replication. Currently, the mechanism of this switch is unknown. However, it is possible that G3BPs/Rin have some role in this process, as the depletion of G3BPs severely reduces negative-strand RNA synthesis in CHIKV-infected cells (208). Therefore, it is possible that the binding of Rin to nsP2 might influence the switching process and prevent the reversal of the lethal phenotype. It is also plausible that there are two effects involved—the binding of Rin to nsP2 has both a pro-viral effect (as observed in experiments with *trans*-replicases) and a negative impact on CHIKV infection (as observed in experiments with mutants based on SP6-CHIKV). As a result, no rescue is observed. Finally, C6/36 cells may not be fully suitable for rescue experiments because these cells are easy to infect but rather difficult to transfect. The low efficiency of transfection may cause false-negative results; i.e., rescue was not observed because only a small number of cells were transfected with RNA transcripts. This number was sufficient to rescue the wt virus and its variants with intact interaction sites in nsP3 but was too low to rescue more strongly attenuated variants of SP6-3^{F3A}CHIKV.

4.3.3. Insertion of the G3BP/Rin-binding motifs after residue 466 or 618 restores replication of 3^{F3A}CHIKV mutants in BHK21 cells

Due to the limitations associated with the transfection of C6/36 cells and to exclude cell type-specific effects, we further checked whether the same set of CHIKV mutants could be rescued in BHK-21 cells, which are easy to transfect and highly susceptible to CHIKV infection. Insertion of the WT peptide after residue 8 of nsP2 did not restore the replication of the 3^{F3A}CHIKV mutant (i.e., the virus in which native G3BP-binding motifs were mutated). In contrast, the WT peptide inserted after residue 466 or 618 of nsP2 could restore the replication of 3^{F3A}CHIKV, as shown by clear capsid protein expression in cells transfected with the corresponding RNA transcripts (**Publication III, Figure 7A**). Although replication was restored for both of these constructs, they had different phenotypes. For the construct with the WT peptide inserted after residue 466, the RNA copy number in the obtained virus stock was below the detection level; consistently, no capsid protein expression could be detected in BHK21 cells infected with the harvested stock (**Publication III, Figure 7B**). These data suggest that although the binding of G3BPs to the WT peptide located after residue 466 could

restore CHIKV genome replication and SG RNA synthesis, this binding likely had a negative effect on virion formation and/or release. In contrast, viral RNA was clearly detected in the stock of the virus in which the WT peptide was inserted after residue 618 of nsP2 and in BHK21 cells infected with this virus, a low level, but clear expression of the capsid protein was detected (**Publication III, Figure 7B**). Coherent with the low level of capsid protein expression, the virus was strongly attenuated and did not cause a cytopathic effect in transfected or infected cells. As we learned in previous studies, such a phenotype is sometimes caused by adaptive mutations. Therefore, we used reverse transcription PCR (RT–PCR) and sequencing analysis to confirm that there were no deletions or adaptive mutations in the HVD or in the 1/2 site, i.e., in the positions proven to generate either deletions or adaptive mutations to restore the replication of CHIKV harboring F3A in the HVD of nsP3 (181). Therefore, the restored replication was indeed due to the insertion of the WT peptide after residue 618 of nsP2.

The original CHIKV mutant with the F3A mutation in the HVD was also able to replicate and generate infectious progeny **(Publication III, Figure 7A, 7B**). Previous studies revealed that at this case, the restoration of infectivity was achieved by a deletion mutation that re-established one of the G3BP binding motifs (181). To find out if this also applied to our study, the RT–PCR and sequencing analysis described above were used. No deletion similar to the previously described deletion was found in the nsP3 region; however, a number of seemingly random deletions were observed in this region. A previous study revealed that CHIKV could restore its replication in U2OS ΔΔ cells or when the G3BP binding motifs in nsP3 were mutated by acquiring an Arg to His substitution at the P4 position of the 1/2 site (181). RT–PCR and sequencing of this region revealed an Arg-to-Gly substitution at the same position. When the Arg-to-His and Arg-to-Gly substitutions were analyzed using a *trans*-replication assay, they were found to have similar activating effects on the activities of the CHIKV replicase harboring the F3A mutation in nsP3. Overall, we concluded that various mutations in the 1/2 site and/or deletions in nsP3 found in our study were responsible for the reacquisition of 3^{F3A}CHIKV infectivity.

In summary, our study showed that the binding of G3BPs to nsP2 can support CHIKV replication and, in at least one case, restore the infectivity of viruses lacking G3BP binding motifs in nsP3. However, why does this restoration occur only for insertions made at certain positions in nsP2? The recent elucidation of the molecular architecture of the replicase core structure may provide some explanations. In the replicase core structure (Figure 6, 12B), the N-terminal region of nsP2 forms an interface between nsP4 and nsP1, this arrangement results in steric hindrance, making binding of G3BPs difficult. Therefore, it is not surprising that we could not detect the replication of 3^{F3A}CHIKV when the WT peptide was inserted after residue 8 in nsP2. In the replicase core structure, the additional density located above nsP2h is assumed to be the nsP2 protease. In addition, a cytoplasmic ring with a cylinder-like density was defined in proximity to the nsP2. The components of this cytoplasmic ring are unknown, but this structure is likely formed by nsP3 and host factors bound to nsP3, including

G3BPs. Therefore, the closer the WT peptide is to the cytoplasmic ring, the more accessible it is to the components of this structure. Therefore, the CHIKV mutant (F3A in nsP3) with the WT peptide inserted after residue 618 can restore the infectivity of the virus and enable it to complete the full replication cycle. However, the virus was strongly attenuated. This difference might be due to the repositioning of G3BPs from the outer cytoplasmic ring to the core structure of the replicase, where only one molecular nsP2 is available. However, the amount of nsP3 subunits in the cytoplasmic ring is unknown; hence, it is unclear whether and how different amounts of G3BP molecules brought to the RNA replicase core affect its activity. As mentioned above, the activating effect can, at least in part, be due to the presence of different complexes, including nsP2, nsP3 and G3BPs. To determine which, if any, of these possibilities are true, one needs to know where and how nsP3 is located in infected cells and what the pro-viral functions of G3BPs are. In conclusion, our study demonstrated that the CHIKV G3BP binding motifs in nsP3 are not strictly needed for the interaction of this replicase component with host G3BPs; the presence of G3BP binding motifs in nsP2 can also support CHIKV replication.

5. CONCLUSIONS

Over the last decade, our understanding of the structures and replication machinery of alphavirus replicases has greatly increased. To date, three-dimensional structures of all individual nonstructural proteins have been obtained. Furthermore, the crystal structure of the alphavirus replicase core was determined, providing the molecular basis for understanding alphavirus RNA replication. Among nonstructural proteins, nsP2 is one of the driving forces essential for successful RNA replication, as it performs the sequential cleavage of nonstructural polyproteins and exhibits RNA helicase, NTPase, and RTPase activities that are essential for viral RNA synthesis. However, the coordination of all these activities and other functions of nsP2 is poorly understood.

The results of the present study provide a structural basis for a clearer understanding of the roles of nsP2 in the alphavirus replication process and indicated that nsP2 can be engineered to perform additional functions that are required for virus replication. The main conclusions of this study are as follows:

1. The structure of the nsP2 helicase (nsP2h) was revealed. Three stacking interactions formed between nsP2h and ssRNA were found to be crucial for CHIKV replication. Mutations of the Y161 or the F164 residue did not have a negative effect on the ATPase activity of the protein, indicating that they are most likely important for RNA helicase activity. F287 is important for the synthesis of SG RNA of virus. Its replacement with alanine is poorly tolerated, and viruses restore their infectivity by acquiring pseudoreversion or second-site compensatory mutations. In addition, the residues of the NTP binding site were crucial for CHIKV replication; mutation of these residues led to the loss of ATPase activity of nsP2.

2. The nsP2h and nsP2 protease (nsP2p) regions are connected by a flexible linker (amino acid residues 463–476). The linker could tolerate the deletion of one amino acid residue and the insertion of up to 10 amino acid residues without influencing viral replication. However, deletions of three or five amino acid residues were lethal for CHIKV.

3. The insertion of the G3BP/Rin binding motifs into the flexible regions of nsP2 increased the activity of CHIKV replicases that were unable to bind G3BP/Rin via nsP3. Similar effects were observed in both human and mosquito cells. The most prominent replication-enhancing effects were observed for replicases with G3BP/Rin binding motifs inserted after residue 618 in nsP2.

4. The binding of G3BPs to nsP2 restored the infectivity of viruses that lacked G3BP binding motifs in nsP3, and this effect was observed in BHK21 cells but not in C6/36 cells. If the G3BP binding site was introduced after residue 466 of nsP2, the mutant failed to produce infectious progeny; however, if the G3BP binding site was introduced after residue 618 of nsP2, the mutant virus was attenuated but able to complete a full replication cycle.

SUMMARY IN ESTONIAN

Struktuuri põhine vaade CHIKV nsP2 valgu funktsioonidele

Viimase aastakümne jooksul on meie arusaam alfaviiruste replikatsiooni mehhanismidest ja seda läbiviivatest valkudest oluliselt suurenenud. Kindlaks on tehtud kõigi nelja mittestruktuurse (replikaasi) valgu ruumilised struktuurid; lisaks on välja selgitatud ka tervikliku replikatsioonkompleksi struktuur. Need saavutused on võimaldanud paremini mõista alfaviiruse RNA replikatsiooni molekulaarseid aluseid. Alfaviiruse replikaasi all-ühikute hulgast on nsP2 üks kõige olulisemaid. Selle valgu proteaasne osa (nsP2p) teostab viiruse mittestruktuurse eelvalgu lõikamist, see protsess on funktsionaalsete replikatsioonikomplekside moodustamise eelduseks. Lisaks omab nsP2 ka RNA helikaasi, NTPaasi ja RNA 5′ γ-fosfataasi aktiivsuseid ning osaleb nakatunud rakkudes viiruste-vastase kaitse mahasurumises. Selle kohta, kuidas toimub nende funktsioonide omavaheline koordineerimine, oli aga vähe informatsiooni. Käesolev väitekiri põhineb uurimistöödel milles selgitati välja nsP2 helikaase regiooni (nsP2h) ruumiline struktuur ja uuriti selle regiooni koostööd nsP2p regiooniga. Lisaks näidati, et niigi polüfunktsionaalsele nsP2-le saab peremees-raku valkude seondumismotiivide lisamise kaudu anda täiendavaid funktsioone. Käesoleva uuringu peamiseid järeldusi saab kokku võtta järgnevalt:

1. nsP2h regioon seondub RNAga „stacking“ interaktsioonide kaudu. Kahe sellistes interaktsioonides osaleva aminohappe jäägi (Y161, F164) muteerumine ei mõjutanud nsP2 valgu NTPaaset aktiivsust kuid blokeeris täielikult viiruse RNA replikatsiooni. See annab alust oletada, et need aminohappe jäägid on olulised RNA helikaasseks aktiivsuseks. Kolmanda „stacking“ interaktsioonis osaleva aminohappe (F287) vahetamine alaniini jäägi vastu vähendas spetsiifiliselt viiruse subgenoomse RNA sünteesi. Sellist mutatsiooni kandev viirus oli nõrgestatud kuid võimeline taas-aktiveeruma pseudoreversiooni ja adaptiivsete mutatsioonide tekitamise kaudu. Lisaks „stacking“ interaktsioonides osalevate aminohappejääkidele olid viiruse replikatsiooniks olulised ka NTP sidumissaiti moodustavad aminohappejäägid K192 ja Q283. Nende aminohappe jääkide muteerumine blokeeris nsP2 NTPaasse aktiivsuse; samuti olid need mutatsioonid viirusele letaalsed.
2. nsP2h ja nsP2p regioonide vahel paikneb linker-domeen (aminohappe jäägid 463–476). See on paindlik regioon mis on oluline CHIKV replikatsiooni jaoks. Ühe aminohappe jäägi deletsioon või kuni 10 aminohappe jäägi lisamine sellese regiooni ei mõjutanud oluliselt viiruse infektsioonilisust. Küll aga põhjustas kolme või viie aminohappe jäägi deletsioon viiruse infektsioonilisuse kadumise.
3. Peremeesraku G3BP/Rin valkude seondamiseks vajaliku motiivi lisamine nsP2 paindlikesse piirkondadesse võimaldas viiruse replikaasil kompenseerida G3BP/Rin ja nsP3 valgu vahelise interaktsiooni puudumist; see efekt oli

sarnane inimese ja sääse rakkudes. Kõige suuremat efekti omasid G3BP/Rin sidumismotiivid mis paigutati nsP2 valku 618-nda aminohappe jäägi järele.

4. G3BP-de sidumine nsP2-ga oli piisav CHIKV RNA replikatsiooni, mida takistas G3BP-nsP3 interaktsioonide puudumine, taas-aktiveerimiseks. Vastavaid mutatsioone sisaldavad genoomid olid infektsioonilised hamstri rakkudes (BHK21) kuid mitte sääse rakkudes (C6/36). Saadud mutantide omaduses sõltusid G3BP-de seondumist võimaldavate järjestuste paiknemisest. Kui need järjestused paigutati nsP2 aminohappe jäägi 466 järele, suutis selline viirus küll replitseeruda kuid ei suutnud toota uut viiruste põlvkonda. Kui G3BPd seondavad järjestused paigutati nsP2 aminohappe jäägi 618 järele, oli saadud viirus küll nõrgestatud kuid suutis siiski läbi viia täieliku paljunemistsükli.

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